

# **Modulators of Toll-like Receptors-7 and -8**

By

**Nikunj M. Shukla**

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**and the**

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Dissertation Committee:

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Chairperson (Sunil A. David)

---

David Moore

---

Apurba Dutta

---

Teruna Siahaan

---

Michael Rafferty

Date defended: \_\_\_\_\_

**The Thesis Committee for Nikunj M. Shukla certifies**

**that this is the approved version of the following thesis:**

**Modulators of Toll-like Receptors-7 and -8**

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---

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---

Teruna Siahaan

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## Abstract

Toll-like receptors (TLR)-7/-8 are innate immune receptors present in the endosomal compartment that are activated by single-stranded RNA (ssRNA) molecules of viral as well as nonviral origin, inducing the production of inflammatory cytokines necessary for the development of adaptive immunity, and are thus useful as vaccine adjuvants. A general introduction to TLRs, with an emphasis on the role of TLR7 activation in mobilizing innate and adaptive immune responses is presented in Chapter 1.

Synthetic small molecule agonists of TLR7 include the imidazoquinoline class of compounds such as Gardiquimod [1-(4-amino-2-((ethylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol]. En route to the synthesis of gardiquimod, its *des*-amino 3*H* regioisomer was found to be antagonistic at TLR7 (Chapter 2).

Using the 3*H* imidazoquinoline as a lead, the syntheses, characterization, and biological evaluation of a pilot library of 3*H* imidazoquinolines was undertaken, which led to the identification of a dual TLR7/TLR8 antagonist (Chapter 3).

With the goal of developing more potent TLR7 agonists as adjuvants, derivatives of gardiquimod were synthesized and a detailed SAR study on the imidazoquinoline chemotype was performed, which led to the discovery of a highly potent, lipophilic, human TLR7 agonist (Chapter 4).

Further exploration on the imidazoquinoline chemotype led us to a highly active TLR7/8 dual agonistic molecule bearing a free primary amine on the *N*<sup>1</sup> substituent. This molecule was utilized to synthesize fluorescent imidazoquinoline analogues that retained TLR7/8-agonistic activity, and were used to study the distribution of TLR7 and also to examine its differential uptake in lymphocytic subsets (Chapter 5).

Homodimers of imidazoquinolines were synthesized in order to test whether such constructs would behave as modulators of TLR3 (Chapter 6).

The TLR7/8 dual agonistic molecule was also used as a convenient precursor for the synthesis of isothiocyanate and maleimide derivatives, enabling its direct conjugation to protein and polysaccharide antigens to make self adjuvanting vaccine constructs. The isothiocyanate derivative was covalently coupled to a model antigen,  $\alpha$ -lactalbumin, and this self-adjuvanting  $\alpha$ -lactalbumin construct induced robust, high-affinity immunoglobulin titers in murine models of vaccination (Chapter 7).

## Abbreviations

AIDS	acquired immune deficiency syndrome
AP-1	activator protein-1
APC	allophycocyanin
APCs	antigen presenting cells
CCR	chemokine receptor
CD	cluster of differentiation
CTL	cytotoxic T lymphocytes
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCs	dendritic cells
DMAP	4-dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DPLA	diphosphoryl lipid A
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EC <sub>50</sub>	half-maximal effective concentration
EDCI.HCl	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA	enzyme linked immunosorbent assay
ESI-TOF	electrospray ionization-time of flight
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FSC	forward scatter
GCOS	genechip operating software
GI	gastrointestinal
GM-CSF	granulocyte macrophage-colony stimulating factor
HATU	2-(1 <i>H</i> -7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate
HBTU	2-(1 <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate
HEK-293	human embryonic kidney 293
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSA	human serum albumin
IC <sub>50</sub>	half-maximal inhibitory concentration
IFN	interferon
Ig	immunoglobulin
IL	interleukin

IRAK4	IL-1-receptor-associated kinase 4
JNK	Jun <i>N</i> -terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
<i>m</i> -CPBA	<i>meta</i> -chloroperoxy benzoic acid
MHC	major histocompatibility complex
MIP-1 $\alpha$	macrophage inflammatory proteins-1alpha
MPLA	monophosphoryl lipid A
MyD88	myeloid differentiation primary response gene (88)
Nalp3	NACHT, LRR and PYD domains-containing protein 3
<i>N</i> -Boc	<i>N-tert</i> -butyl carbamate
NF- $\kappa$ B	nuclear factor-kappa B
NK cells	natural killer cells
NOD	nucleotide-binding oligomerization domain
NOE	nuclear overhauser effect
PAMP	pathogen associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PE	phycoerythrin
RIG	retinoic acid inducible gene
RNA	ribonucleic acid
sAP	secreted alkaline phosphatase
SAR	structure activity relationship
S <sub>N</sub> Ar	aromatic nucleophilic substitution
SSC	side scatter
ssRNA	single stranded RNA
TGF- $\beta$	transforming growth factor beta
Th1	helper T-type 1
THF	tetrahydrofuran
TIR	toll-interleukin 1 receptor domain
TLRs	toll like receptors
TNF- $\alpha$	tumor necrosis factor – alpha
Tregs	human T-regulatory cells
TRIF	toll-interleukin 1 receptor domain-containing adapter-inducing interferon- $\beta$

## Chapter 1.

# INTRODUCTION





## 1.1. Introduction – Adjuvants, Toll-like Receptors (TLRs), and Other Microbial Sensors.

*As interesting as this method is from a practical point of view, it may be just as interesting from a theoretical point of view, because of the research it could stimulate to understand the intimate mechanism of either the increase in antitoxin induced in this case, or the generation of antitoxins within the animal”*

*Gaston Ramon, 1926*

Just as the prescient comment by Gaston Ramon was relegated to the last footnote of his 1925 paper,<sup>1</sup> so has research on the mechanisms of action of adjuvants, until recently, languished as parenthetical annotations and addenda in the archives of immunology and vaccine development. Ramon defined immunological adjuvants as “*substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone.*” Interestingly enough, he was referring to his empirical findings that the addition of bread crumbs, tapioca, saponin, and ‘starch oil’ to antigenic preparations greatly enhanced antibody responses to diphtheria or tetanus.<sup>2</sup> A year later, the adjuvanticity of aluminum salts (primarily phosphate and hydroxide) was discovered by Glenny and coworkers.<sup>3</sup> In the eighty-one years that have elapsed, the repertoire of investigational adjuvants has grown to encompass a very wide range of materials,<sup>4</sup> but aluminum salt-based mineral salts (generically, and incorrectly, termed “alum”) have remained the only adjuvants currently approved by the FDA. Aluminum salts have enjoyed a good safety record, but they are weak adjuvants for antibody induction and induce a Th2-skewed, rather than a Th1 response.<sup>5,6</sup> Furthermore, not only are aluminum salts ineffective at inducing cytotoxic T lymphocyte (CTL) or mucosal IgA antibody responses, but also have a propensity to induce IgE responses, which have been associated with allergic reactions in some subjects.<sup>5,6</sup> Very recent reports implicate the Nalp3 inflammasome, a component of the innate immune response, as the effector limb of alum-associated adjuvanticity.<sup>7-9</sup>

In 1962, Dresser observed that injection of purified soluble proteins not only failed to stimulate an immune response, but tolerized animals unless a *bacterial extract* was admixed with the protein immunogen.<sup>10-12</sup> This led him to redefine adjuvanticity “as a property of a substance which can act as a physiological switch, directing at least some immunologically competent cells to respond by making antibody rather than by becoming immunologically paralyzed by the antigen”,<sup>13</sup> confirming Johnson’s earlier observations that lipopolysaccharide from Gram-negative bacteria exerted potent adjuvant properties,<sup>14</sup> and perhaps paved the way for the subsequent discovery of the wide range of microorganism-derived adjuvants shown in **Table 1**.<sup>15</sup>

**Table 1.** Adjuvants and their usage of innate immune receptors.

*Reproduced from Ref. 15.*

Adjuvant	Innate immune receptor	Ligand component	Source (origin)
Pam <sub>3</sub> Cys-SK <sub>4</sub>	TLR2 and TLR1	Lipoprotein	Bacteria or synthetic
MALP-2	TLR2 and TLR6	Lipopeptide	Bacteria or synthetic
OspA	TLR2 + unknown	Bacterial cell wall	<i>Borrelia burgdorferi</i>
Hib-OMPC			<i>Haemophilus influenzae</i> type b
PolyI:C	TLR3	dsRNA	Virus or synthetic
MPL (Monophosphoryl-lipid A/trehalose dicorynomycolate (“Ribi” adjuvant))	MDA5		
Flagellin	TLR4 + unknown	LPS + unknown	Gram negative bacteria
	TLR5	Flagellin	Bacteria
Imidazoquinolins	IPAF		
Polyuridylic acid (poly-U)	NAIP5		
CpG ODN	TLR7/8	Synthetic RNA analogs	Virus or synthetic
Hemozoin	TLR7/8 + unknown	ssRNA	
	TLR9	Unmethylated CpG motifs	Bacteria or synthetic
	TLR9	Hemozoin	<i>Plasmodium falciparum</i> or Synthetic
Plasmid DNA	TLR9 + unknown	CpG motifs + unknown	Bacteria
iE-DAP (γ-d-glutamyl-meso-DAP)	NOD1	Desmuramylpeptides (DMP) containing diaminopimelic acid (DAP) within	Bacteria or synthetic
FK565 and FK156	NOD1	Peptidoglycan	
Complete Freund’s adjuvant (CFA)	NOD2 + unknown (TLR2, 4?)	Muramyl dipeptide (MDP)	Bacteria
?	NALP3/cryopyrin/CIAS	Muramyl dipeptide	N/A
		Toxins	
		Bacterial RNA	
		Uric acid crystals	

While it is indisputable that progress in developing adjuvants had been stymied due to the lack of a detailed understanding of the mechanistic bases underlying their immunopotentiatory properties as well as the pronounced systemic toxicity of bacterial products, it is astonishing that purely empirical observations (which predate by many decades the discovery of what is taken for granted today, such as the professional antigen presenting cell, or the immunological synapse) had fueled incremental advances, driving the field slowly to a point where it is now poised to reach ‘critical mass’ due to the convergence of the decade-old science of innate immunity,<sup>16-20</sup> and the nascent field of toll-like

receptors (TLRs),<sup>15;21-25</sup> and other Pathogen-Associated Molecular Pattern (PAMP)-recognizing elements such as the nucleotide-binding oligomerization domain (NOD)-like<sup>26-31</sup> and retinoic acid inducible gene (RIG)-like receptors.<sup>32-36</sup> Safety and the lack of toxicity, alongside efficacy are, obviously, the central issues in the choice of a vaccine adjuvant. In the 1950s, Salk vaccinated humans with influenza virus emulsified in Freund's incomplete adjuvant (water-in-oil emulsion of mineral oil and the surfactant mannide monooleate). Based on the reported safety of the adjuvant in influenza vaccine trials,<sup>37</sup> other investigators explored Freund's incomplete adjuvant (Montanide ISA 720 and ISA 51<sup>38</sup>) for use in single dose vaccines. A high incidence of severe local reactions, however, led to an abrupt termination of the trials.<sup>39;40</sup> Clinical trials on Isatoribine<sup>41-43</sup> (ANA975), a TLR7 agonist, have recently been terminated because of toxicity in animal models.<sup>44</sup> The search for immunopotentiators with a wide margin of safety continues, but the pace of discovery, until very recently, had been hampered by the lack of understanding of the structure-function correlates underlying immunostimulatory versus inflammatory activities.

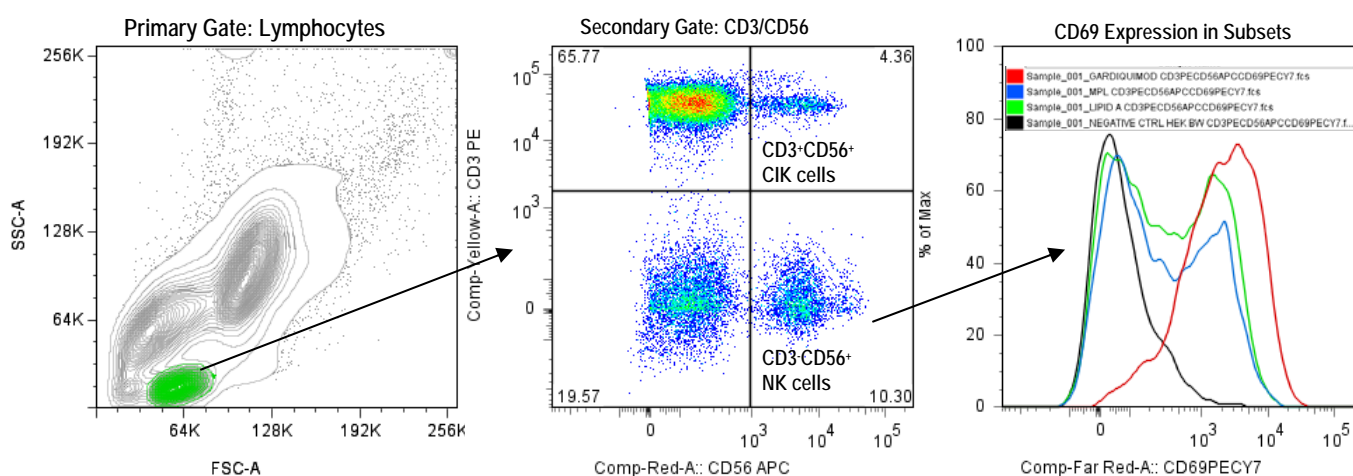
Toll-like receptors (TLRs) are pattern recognition receptors present on diverse cell types. TLRs recognize specific molecular patterns present in molecules that are broadly shared by pathogens but are sufficiently different so as to be distinguishable from host molecules, and are collectively referred to as pathogen-associated molecular patterns (PAMPs).<sup>24;45</sup> There are 10 TLRs in the human genome; these are trans-membrane proteins with an extracellular domain having leucine-rich repeats (LRR) and a cytosolic domain called the Toll/IL-1 receptor (TIR) domain.<sup>45</sup> The ligands for these receptors are highly conserved microbial molecules such as lipopolysaccharides (LPS) (recognized by TLR4), lipopeptides (TLR2 in combination with TLR1 or TLR6), flagellin (TLR5), single stranded RNA (TLR7 and TLR8), double stranded RNA (TLR3), CpG motif-containing DNA (recognized by TLR9), and profilin present on uropathogenic bacteria (TLR11).<sup>46;47</sup> TLR1, -2, -4, -5, and -6 respond to extracellular stimuli, while TLR3, -7, -8 and -9 respond to intracytoplasmic PAMPs, being associated with the endolysosomal compartment.<sup>45</sup> The activation of TLRs by their cognate ligands leads to production of inflammatory cytokines, and up-regulation of MHC molecules and co-stimulatory signals in antigen-presenting cells as well as activating natural killer (NK) cells (innate

immune response), in addition to priming and amplifying T-, and B-cell effector functions (adaptive immune responses).<sup>22;23;48;49</sup> Thus, TLR stimuli serve to link innate and adaptive immunity<sup>48</sup> and can therefore be exploited as powerful adjuvants in eliciting both primary and anamnestic immune responses.

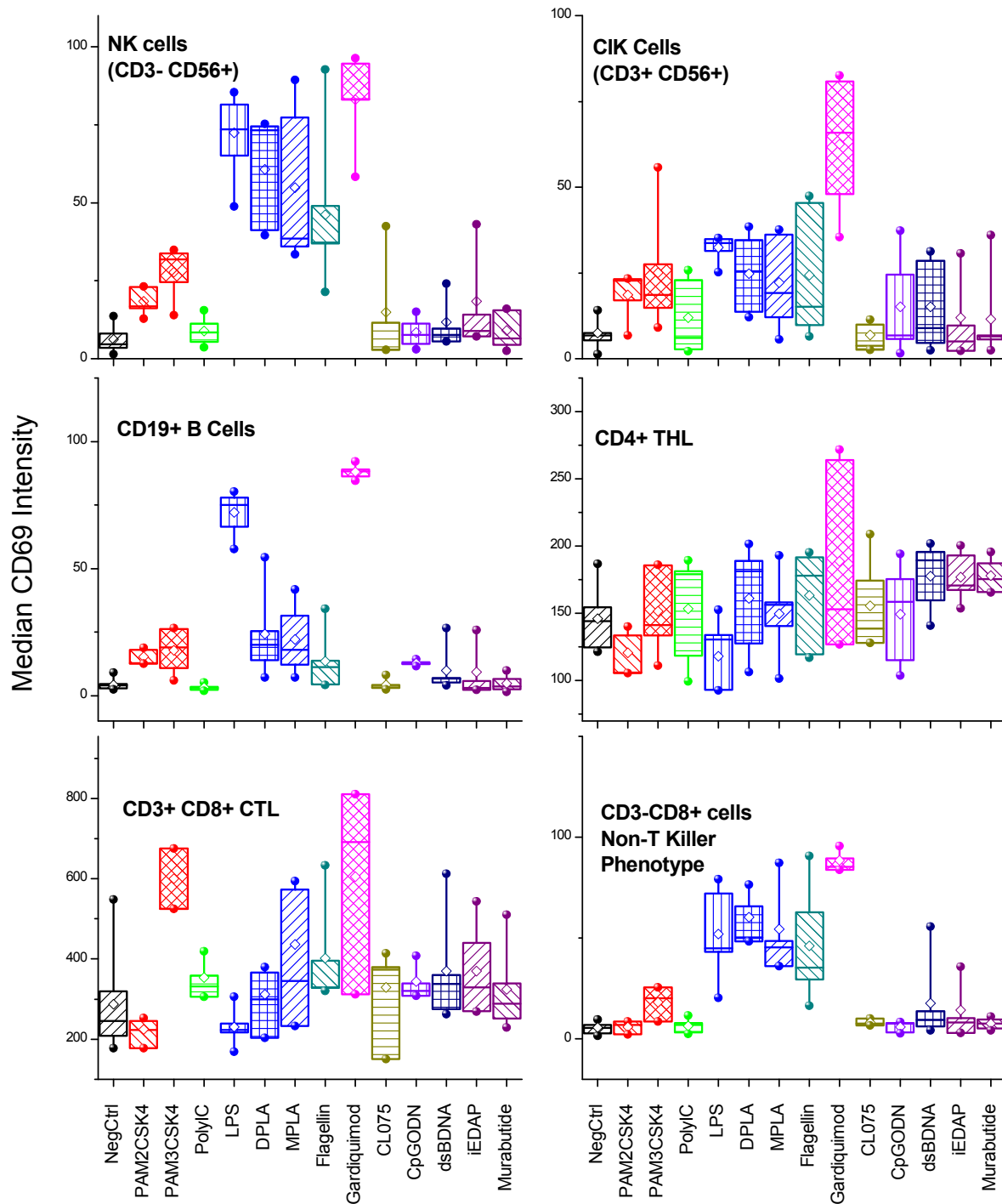
A detailed understanding of the structural and mechanistic bases of adjuvanticity and toxicity is pivotal in rationally developing novel adjuvants. The progression from early experiments demonstrating the adjuvanticity of highly toxic LPS<sup>14</sup> to Ribi's 'detoxified' monophosphoryl lipid A (MPLA) in the 1980s,<sup>50-54</sup> culminating in commercialization as MPL™ in such vaccines as Fendrix® and Cervarix® is instructive, and the lessons learned can be successfully applied toward the rapid development of other TLR agonistic adjuvants. Kusumoto's landmark total synthesis of lipid A, the toxic moiety of endotoxic LPS,<sup>55</sup> allowed the formal verification that synthetic, 1-dephospho-lipid A, as well as 3-deacyl monophosphoryl lipid A isolated by mild acid hydrolysis of Gram-negative bacteria-derived lipid A, were nontoxic, and yet immunostimulatory and adjuvantic.<sup>50;56;57</sup> The mechanism of immunopotentiality of LPS and lipid A, however, remained obscure until the discovery of TLR4-agonistic effects of LPS and lipid A derivatives in the late 1990s,<sup>23;58-63</sup> and it was not until 2007 that the basis of the dissociation between toxicity of diphosphoryl lipid A (DPLA) on the one hand, and the potent adjuvanticity (and nontoxicity) of monophosphoryl lipid A, on the other, became apparent. Whereas DPLA, upon binding to TLR4, signals via both myeloid differentiation factor 88 (MyD88) as well as Toll-interleukin 1 receptor domain-containing adapter inducing interferon- $\beta$  (TRIF)-dependent pathways,<sup>24;64-67</sup> MPLA appears to predominantly trigger the TRIF-dependent pathway.<sup>68</sup> Activation of MyD88-dependent signaling is thought to be associated with proinflammatory outcomes, while activation of TRIF-dependent pathways are correlated with immunostimulation.<sup>68</sup>

In an effort to explore and develop strategies for the rapid and simultaneous evaluation of both adjuvantic potency and proinflammatory activities of relatively large focused libraries of compounds being generated by our synthetic programs, we have examined representative members of virtually the entire compendium of known TLR agonists in a series of hierarchical assays including primary TLR-reporter assays, secondary indices of immune activation such as cytokine induction and activation of lymphocytic subsets in whole human blood, and tertiary screens characterizing transcriptomal activation patterns with a view to identifying optimal immunostimulatory chemotypes. Of all the innate immune stimuli examined, we found that TLR2 (thioacylglycerol lipopeptide chemotype), TLR4 (LPS and monophosphoryl lipid A), TLR5 (flagellin), and TLR7 (imidazoquinoline chemotype) were immunostimulatory; the imidazoquinoline class of TLR7 agonists was found to be extraordinarily immunostimulatory, stimulating virtually all subsets of lymphocytes, and yet without inducing dominant proinflammatory cytokine responses (unlike TLR8 agonists, for instance, which, like LPS, were proinflammatory and therefore may exert systemic toxicity; **Figs 1, 2**).<sup>69</sup>

**Fig. 1.** Schematic for flow cytometric analyses of CD69 expression in various lymphocytic subsets in whole human blood stimulated with 1  $\mu\text{g/mL}$  of various TLR agonists. Primary gating was performed on FSC/SSC-gated lymphocytes. Fully-compensated CD3/CD56 subsets could be resolved with ease, and CD69 expression was measured in each of the subsets. Similar three-color experiments were also performed with CD3, CD4/CD8/CD19, and CD69 combinations. Data from Ref. 69.



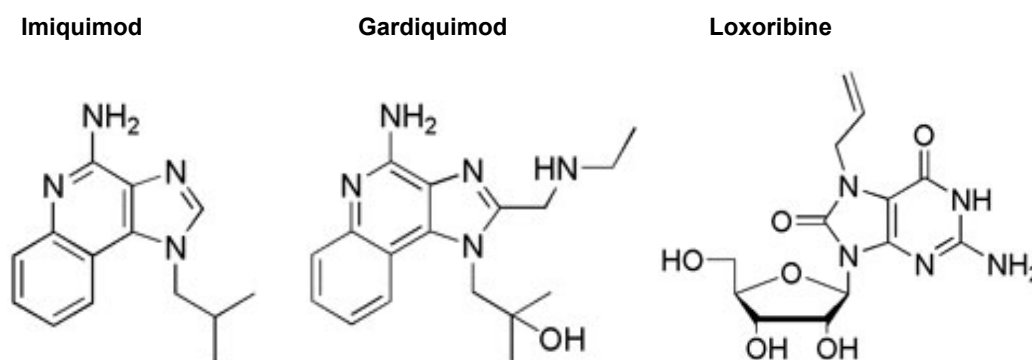
**Fig. 2.** CD69 expression in lymphocytic subsets in whole human blood stimulated with TLR agonists (box-and-whisker plots). Analysis of data obtained from five independent experiments using blood donated by six different donors. Data from Ref. 69.



## 1.2. Intracellular TLR7 Activation: Imidazoquinoline ligands:

Single-stranded RNA (ssRNA) molecules of viral as well as nonviral origin (poly dT; thymidine homopolymer phosphorothioate) induce the production of inflammatory cytokines, mediated by the recognition in the endosomal compartment by TLR7.<sup>70-72</sup> Small molecule, non-polymeric, synthetic agonists include the imidazoquinolines (Imiquimod, Resiquimod [R-848], and Gardiquimod),<sup>73-75</sup> as well as guanosine analogues such as loxoribine<sup>76-78</sup> (**Fig. 3**)

*Fig. 3. Representative synthetic, non-polymeric TLR7 agonists.*



Long before the fact that these synthetic analogues specifically engaged and activated TLR7 was known, it was recognized that these compounds were potently adjuvantic<sup>76-82</sup> in addition to displaying anti-viral effects via the induction of IFN- $\alpha/\beta$ .<sup>70;75;83;84</sup> It became apparent as early as 1991 that loxoribine (7-allyl 8-oxoguanosine) potentiated anti-tetanus-specific IgG antibody anamnestic responses in a dose-dependent manner in human peripheral blood mononuclear cells,<sup>78</sup> as well as strongly activating NK cells in an IL-12-dependent manner.<sup>76;79;80</sup> In human blood stimulated *ex vivo* with TLR7 agonists, upregulation of the surface expression of costimulatory molecules such as CD40, CD80, and CD86 occurred in both CD11c<sup>-</sup> plasmacytoid DCs and CD11c<sup>+</sup> myeloid DCs; furthermore, the Th1 stimulatory ability of both DC subsets was enhanced in response to TLR7 ligands.<sup>85</sup> Because TLR7 agonists transmit a T-helper-like signal to antibody-producing B cells, it is a highly effective adjuvant even for synthetic peptides that lack T-cell epitopes, possibly even replacing the function of T-helper cells, and providing a potential T-cell-independent vaccination strategy.<sup>81</sup>

One potential drawback is that small molecules such as the imidazoquinolines quickly diffuse out of the vaccination site, thereby limiting their utility as an adjuvant. This has been addressed by conjugating the TLR7 ligand to the immunogen(s) of interest. For instance, non-human primates immunized with HIV Gag protein-TLR7 agonist conjugate showed dramatic enhancement of the magnitude and the quality of Th1 responses, as well as eliciting Gag-specific CD8<sup>+</sup> CTL responses compared with animals immunized with HIV Gag protein and the TLR7 agonist as a noncovalent mixture.<sup>86</sup> A number of preclinical studies with TLR7 agonists as adjuvants are currently in progress.<sup>87-92</sup>

### **1.3. Intracellular TLR8 Activation: Imidazoquinoline and Thiazoloquinolone ligands:**

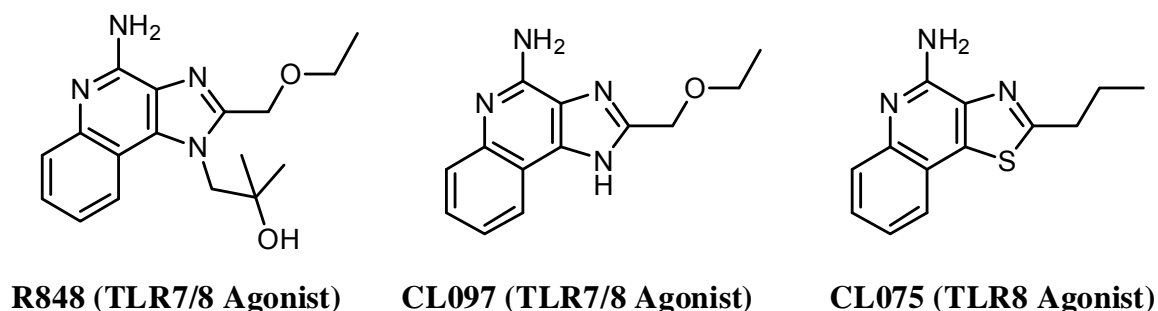
TLR7 and TLR8 are phylogenetically and structurally related and, like TLR7, single-stranded RNA (ssRNA) molecules are recognized in the endosomal compartment also by TLR8, but significant species specificities exist: murine TLR7 and human TLR8 mediate the recognition of GU-rich ssRNA.<sup>93</sup> In human cells, TLR8 agonists activate myeloid dendritic cells, monocytes, and monocyte-derived dendritic cells, with the production of GM-CSF/IL-4/TGF-beta. TLR7-selective agonists were found to be more effective than TLR8-selective agonists at inducing IFN- $\alpha$  and IFN- $\gamma$ -regulated chemokines, whereas TLR8 agonists induce a dominant proinflammatory cytokine profile including TNF- $\alpha$ , IL-12, and MIP-1 $\alpha$ .<sup>94</sup> Not surprisingly, several imidazoquinolines have mixed TLR7/8 agonistic activity, but a thiazoloquinolone compound (3M-002) is characterized as a pure TLR8 agonist (**Fig. 4**).<sup>95</sup> The prominent NF- $\kappa$ B- and JNK-mediated stimulatory effects of TLR8-agonists on antigen-presenting cells<sup>95;96</sup> as well as the activation of NK cells,<sup>97</sup> leads to robust IL-12-driven Th1-type responses.<sup>98</sup>

Studies of human neonatal APCs cultured *in vitro* have demonstrated that TLR7/8 agonists possess unique efficacy to induce the Th1-polarizing cytokine TNF- $\alpha$  at both the transcriptional and translational levels.<sup>98</sup> TLR8 agonists, including the imidazoquinoline congeners R848 (TLR7/8) and



3M-003 (TLR7/8), the amidinothiazoline 3M-002 (TLR8), as well as ssRNAs (TLR8) induce a marked production of the Th1-polarizing cytokines TNF- $\alpha$  and IL-12 from neonatal APCs that substantially exceeds responses induced by TLR2, -4, or -7 agonists.<sup>98</sup> TLR7/8 agonists are thus uniquely efficacious in activating co-stimulatory responses in neonatal APCs, suggesting that these agents are promising candidate adjuvants for enhancing immune responses in newborns.

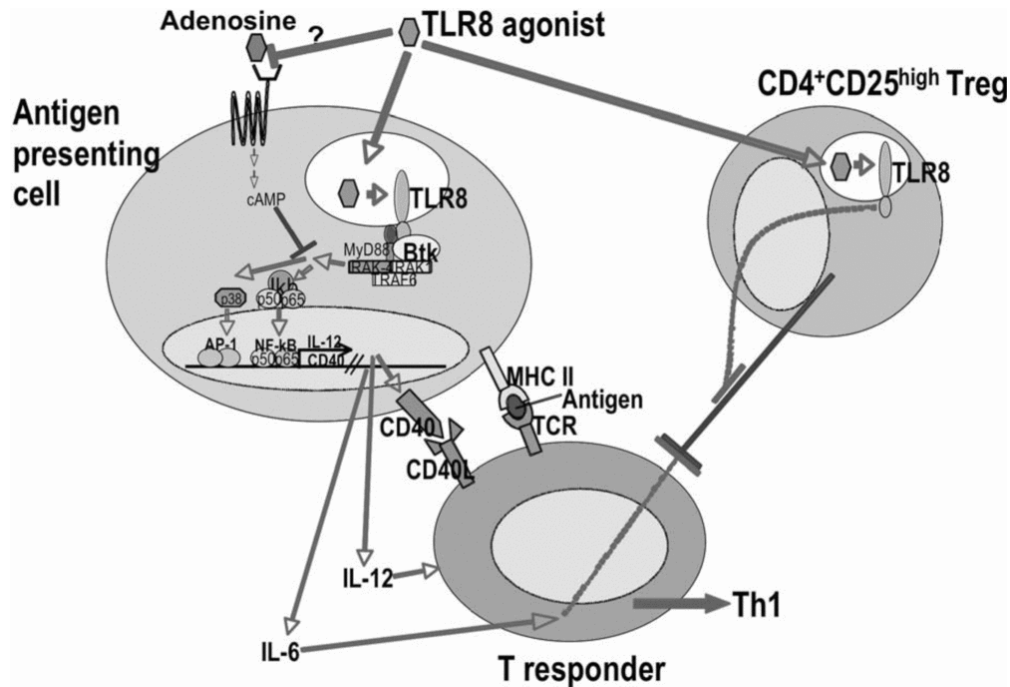
**Fig. 4.** Representative structures of synthetic, non-polymeric, mixed TLR7/8 imidazoquinoline agonists and a pure thiazoloquinolone TLR8 agonist.



#### ***Reversal of Treg suppressive effects by TLR8 agonists:***

Human T-regulatory cells (Tregs), classified immunophenotypically as naturally occurring (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) or induced (CD4<sup>+</sup>CD25<sup>high</sup>), down-regulate and suppress a broad array of immune responses, including the non-specific suppression of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells via cell-cell contact and via production of immunosuppressive cytokines such as IL-10 and TGF- $\beta$ .<sup>99-105</sup> Tregs express abundant TLR8 mRNA, and TLR8 agonists have been shown to reverse Treg function via a TLR8-MyD88 (myeloid differentiation factor 88)-IRAK4 (IL-1-receptor-associated kinase 4) signaling pathway.<sup>106</sup> Engagement and activation of TLR8, therefore, strongly induces innate immunity and enhances adaptive immunity (**Fig. 5**). In addition to the direct effects on Tregs described above, the production of IL-6 in the milieu renders CD4<sup>+</sup> cells refractory to Treg suppression.<sup>107</sup>

**Fig. 5.** TLR8 agonists strongly activate human APCs to induce protective Th1-type immune responses, including production of IL-12 and upregulation of the co-stimulatory molecule CD40. TLR8 agonists also reverse suppression mediated by human Treg cells, via both direct action on Treg as well as by induction of IL-6, which renders responder T cells refractory to Treg-mediated inhibition. Reproduced with modifications from Ref. 106.



**Potential problems with pure, high-potency TLR8 agonists, and a case for a mixed TLR7/TLR8 activator:**

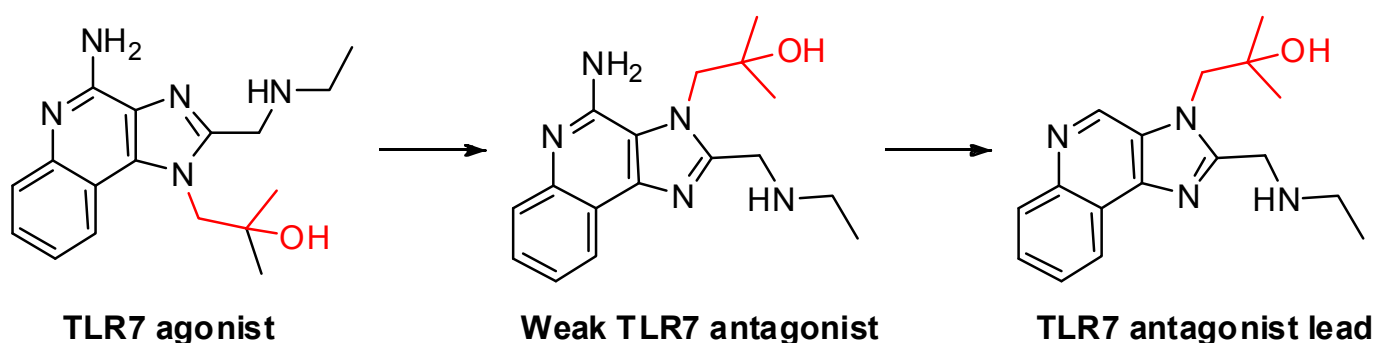
Pure TLR8 agonists are perhaps unique in their ability to very strongly induce the production of proinflammatory cytokines such as TNF- $\alpha$ . This property is thought to be related to the ability of the TLR8 pathway to strongly induce TLR-mediated intracellular signaling via the p38 MAPK and NF- $\kappa$ B pathways.<sup>98</sup> We have confirmed that 3M-002 (CL-075) induced a very potent and sustained proinflammatory response in human whole blood (TNF- $\alpha$ , IL-6, and IL-8), which is not paralleled by a commensurate activation of NK cells or the production of type I or type II interferon, while the converse is true with a pure TLR7 agonist. Local and/or systemic toxicity is to be anticipated and is

therefore a potential concern with potent TNF- $\alpha$ -inducing molecules; indeed such an effect appears to have been contributory to the GI toxicity that has been observed with clinical trials on Isatoribine given orally.<sup>41;42;108</sup>

Based on our preliminary results,<sup>69</sup> we propose that a mixed TLR7/TLR8 agonist, with balanced Th1/IFN/NK cell activating profiles, but with attenuated proinflammatory cytokine induction potency may be beneficial. An extensive synthetic program that adequately explores chemical space around the TLR7/8 chemotype which is coupled optimally to a panel of bioassays should guide not only a careful evaluation of structure-activity correlates in such molecules, but will also likely yield useful molecules with potent adjuvanticity, and with a very wide margin of safety.

## Chapter 2.

# REGIOISOMERISM-DEPENDENT TLR7 AGONISM AND ANTAGONISM IN AN IMIDAZOQUINOLINE



## 2.1. Introduction.

Chronic immune activation is a hallmark of progressive HIV infection which is strongly associated with dysregulated cellular and humoral immune responses;<sup>109;110</sup> furthermore, the resulting accelerated turnover of CD4<sup>+</sup> lymphocytes provides a milieu for HIV replication.<sup>111</sup> The majority of the lymphocytic pool resides in the gastrointestinal tract and it has recently been demonstrated that during acute HIV infection, a tremendous depletion submucosal CD4<sup>+</sup> lymphocytes occurs as a result of massive viral replication.<sup>112-114</sup> The loss of mucosal integrity due to the resultant enteropathy has been shown to contribute to sustained systemic immune activation as a consequence of translocation of bacterial products such as lipopolysaccharide,<sup>115</sup> an agonist of toll-like receptor 4 (TLR4).<sup>116</sup> The engagement of TLR7 and -9 by single-stranded viral RNA has recently been reported to play a central role in immune activation-driven HIV replication. For instance, progressive CD4<sup>+</sup> T lymphocyte depletion in non-human primates is highly correlated with TLR7-mediated interferon- $\alpha$  (IFN- $\alpha$ ) by plasmacytoid dendritic cells, and antagonists of TLR7 inhibit immune activation.<sup>117</sup>

The only known class of TLR7 antagonists is single-stranded phosphorothioate oligonucleotides,<sup>118</sup> and the availability of a selective and potent small-molecule TLR7 antagonist should allow the formal testing of potential benefits of suppression of TLR7-mediated immune activation in HIV/AIDS. Gardiquimod is a commercially-available *N*<sup>1</sup>-substituted 1*H*-imidazoquinoline TLR7 agonist,<sup>119</sup> the synthesis of which had not been published. We undertook the syntheses of gardiquimod and en route, its 3*H* regioisomer as well (**Scheme 1**); the 3*H* regioisomer was found to be completely inactive as a TLR7 agonist and was, in fact, weakly antagonistic. A *des*-amino precursor of the 3*H* regioisomer was more potent as a TLR7 antagonist, with an IC<sub>50</sub> value of 7.5  $\mu$ M. This class of compound may serve as a useful lead and a starting point for the development of small-molecule inhibitors of TLR7. Gardiquimod was also synthesized, and its activity was found to be identical to that of the commercially available compound.

The reaction scheme illustrates the synthesis of Gardiquimod and its regioisomers. It begins with the conversion of 2-aminobenzoic acid (1) to 2-nitrobenzoic acid (2) in 85% yield (step i), followed by cyclization to 2-nitro-3-hydroxyquinoline (3) in 40% yield (step ii). Compound 3 is then converted to 2-nitro-3-chloroquinoline (4) in 75% yield (step iii). From compound 4, two main pathways are shown:

- Left Pathway (Red):** Compound 4 is converted to 2-nitro-3-chloro-4-nitroquinoline (10) in 97% yield (step iv). Compound 10 is then converted to 2,4-diaminoquinoline (11) in 95% yield (step v). Compound 11 is cyclized to 2,4-diamino-3,4-dihydroquinoline (12) in 60% yield (step vi). Compound 12 is then converted to 2,4-diamino-3,4-dihydroquinoline-3-yl tert-butyl ether (13) in 70% yield (step vii). Compound 13 is cyclized to 2,4-diamino-3,4-dihydroquinoline-3-yl tert-butyl ether (14) in 72% yield (step viii). Finally, compound 14 is converted to 3H-regioisomer (15) in 72% yield (step ix).
- Right Pathway (Green):** Compound 4 is converted to 2-nitro-3-chloro-4-aminoquinoline (5) in 72% yield (step x). Compound 5 is converted to 2-amino-3-chloro-4-aminoquinoline (6) in 96% yield (step xi). Compound 6 is converted to 2-amino-3-chloro-4-aminoquinoline-3-yl tert-butyl ether (7) in 80% yield (step xii). Compound 7 is cyclized to 2-amino-3-chloro-4-aminoquinoline-3-yl tert-butyl ether (8) in 82% yield (step xiii). Finally, compound 8 is converted to Gardiquimod (9) in 70% yield (step xiv).

A central box shows the synthesis of compound 18 from compound 17 (80% yield, step xv) and the synthesis of compound 22 from compound 19 (57% yield, step xvi), followed by steps xvii (79% yield) and xviii (79% yield) to compound 22. Compound 18 is used in the synthesis of compound 15 (72% yield, step xix) and compound 16 (72% yield, step xix). Compound 22 is used in the synthesis of compound 16 (72% yield, step xix).

**3H-REGIOISOMER** (15) and **4-Desamino-3H-Regioisomer** (16) are shown as side products. **GARDIQUIMOD** (9) is the final product.

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## 2.2. Results and Discussion.

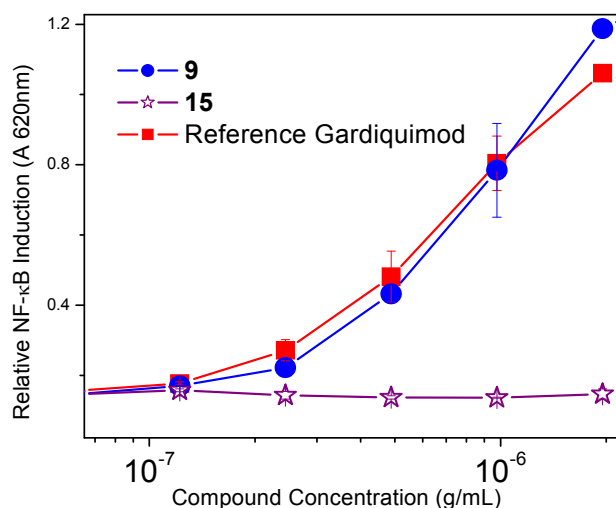
Following literature precedents,<sup>120</sup> anthranilic acid **1** was condensed with 2-nitroacetaldehyde oxime which was prepared *in situ* from nitromethane and sodium hydroxide to give 2-(2-nitro ethylideneamino)benzoic acid, **2**; this was then cyclized to form the quinoline ring using acetic anhydride and potassium acetate to give 3-nitro-4-hydroxyquinoline, **3**. Treatment with phosphorous oxychloride afforded 3-nitro-4-chloroquinoline, **4**, which served as the common precursor for the synthesis of gardiquimod, as well as its regioisomer. 1-amino-2-methylpropan-2-ol, **18** was obtained by reacting NH<sub>4</sub>OH with 2,2-dimethyloxirane at 0 °C; isolation and purification were facilitated by temporary *N*-Boc protection, which was eventually deprotected with CF<sub>3</sub>CO<sub>2</sub>H. The *N*-Boc-protected *N*-ethylglycine derivative **22**, was synthesized from the methyl ester of glycine **19** via reductive amination using acetaldehyde and sodium cyanoborohydride, followed by *N*-Boc protection using (Boc)<sub>2</sub>O and subsequent ester hydrolysis by LiOH.

**Synthesis of gardiquimod:** An S<sub>N</sub>Ar reaction on **4** with 1-amino-2-methylpropan-2-ol, **18** afforded the 3-nitro-4-aminoquinoline derivative **5**, which was converted to the imidazoquinoline **7** via sequential Pd-catalyzed reduction of the nitro group **6**, amidation with the *N*-Boc-protected amino acid **22**, followed by cyclization with NaOH to afford the intermediate **7**.<sup>121</sup> *N*-oxidation of the quinoline nitrogen of **7** with *m*-CPBA yielded **8**, which was reacted with benzoyl isocyanate in dichloromethane to provide a 4-*N*-benzoyl derivative. This amide was cleaved using NaOMe in MeOH as has been reported<sup>122</sup> to yield the *N*-Boc-protected precursor of gardiquimod. Final deprotection was performed with CF<sub>3</sub>CO<sub>2</sub>H to give the target molecule, **9**, as the TFA salt, which was as active as the commercially-available reference compound (**Fig. 1**, overleaf).

**Synthesis of the 3H regioisomer of gardiquimod:** An S<sub>N</sub>Ar displacement of **4** to the corresponding azido derivative **10** with NaN<sub>3</sub>, followed by reduction of both the 3-nitro- and the 4-azido groups with Pd/C under hydrogenation conditions gave the 3, 4-diaminoquinoline **11**. Amidation of **11** with **22**, followed by cyclization using NaOH afforded the *C*-2-substituted imidazoquinoline scaffold **12**.

Reaction of **12** with excess of the 2,2-dimethyloxirane in the presence of DBU afforded the *N*-3 substituted imidazoquinoline **13**. Subsequent steps, similar to the ones described above afforded the 3*H* regioisomer **15**.

**Fig. 1.** NF- $\kappa$ B induction activities of **9** and reference gardiquimod in a TLR7-specific reporter gene assay.  $IC_{50}$  values of both compounds is 0.65  $\mu$ g/mL. **15** was inactive at the highest concentration test (25  $\mu$ g/mL)



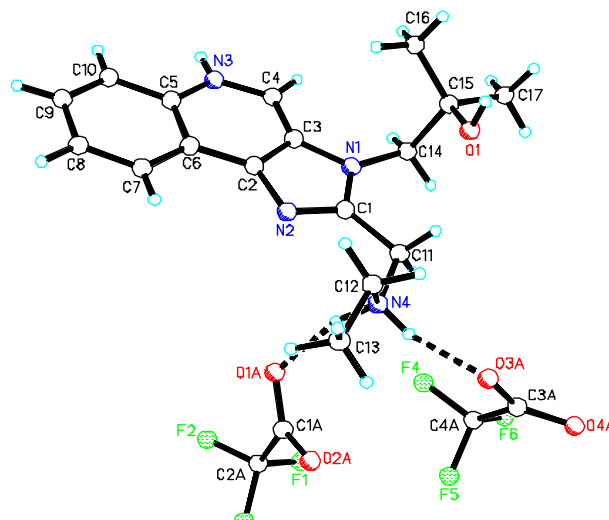
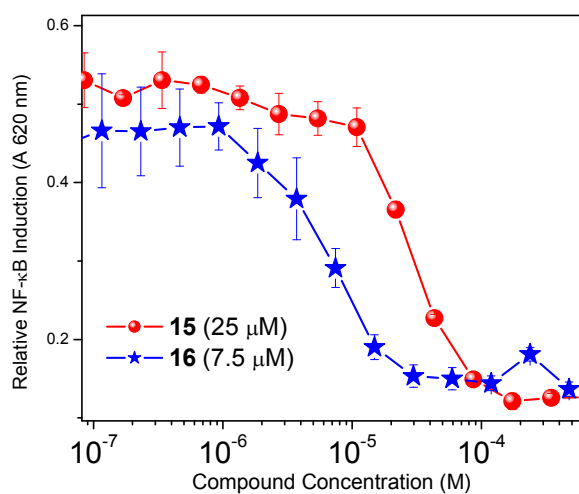
**TLR7 agonism and antagonism:** A reporter gene assay using TLR7-dependent NF- $\kappa$ B induction was used. The induction of NF- $\kappa$ B was quantified using HEK-Blue-cells as previously described by us.<sup>123</sup> HEK293 cells were stably transfected with plasmids encoding TLR7 as well as an NF- $\kappa$ B reporter gene coupled to secreted alkaline phosphatase (sAP) (InvivoGen, San Diego, CA), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF- $\kappa$ B/AP-1 promoters is inducible by the TLR7 agonists, and extracellular sAP in the supernatant is proportional to NF- $\kappa$ B induction. HEK-Blue-7 cells were incubated at a density of  $\sim 10^5$  cells/mL in a volume of 80  $\mu$ L/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm. The TLR7 agonistic potency of **9** was indistinguishable from that of the



commercially available gardiquimod (**Fig. 1**), while **15** was devoid of agonistic activity (**Fig. 1**). None of the precursors of **9** displayed any significant agonistic activities (data not shown).

Antagonistic activities were examined by incubating HEK-Blue-7 cells with graded concentrations of test compounds in the presence of 250 ng/mL of gardiquimod. **15** displayed weak TLR7 antagonism (25  $\mu$ M, **Fig. 2**), which prompted us to carefully examine all its precursors. The *des*-amino compound **16** obtained after deprotecting **13** was found to be maximally antagonistic with an  $IC_{50}$  of 7.5  $\mu$ M. Compound **16**, fortuitously, also crystallized with ease in MeOH, the structure of which is shown in **Fig. 2**.

**Fig. 2.** Left: TLR7-antagonistic activities of the 3*H* regioisomer **15**, and of its *des*-amino precursor **16**. Right: Crystal structure of **16** as the di-TFA salt. The locations of all the hydrogens were determined from a difference Fourier and refined as individual isotropic atoms.



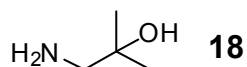
### 2.3. Conclusions.

Compound **16**, was the first small molecule with TLR7-antagonistic activity, and a focused library has been generated for this 3*H*-imidazoquinoline chemotype toward the generation of more potent small-molecule TLR7 receptor antagonists (Chapter 3). Compound **9** i.e. gardiquimod was further derivatized and SAR has been studied in Chapter 4.

## 2.4. Experimental.

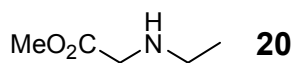
Unless otherwise mentioned, the compounds synthesized were obtained as solids.

### Synthesis of Compound 18: 1-amino-2-methylpropan-2-ol.

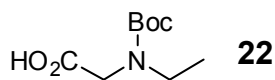


The literature compound **18** was synthesized as reported previously (Close, W.J. *J. Am. Chem. Soc.* **1951**, 73, 95-98). 2,2-Dimethyloxirane **17** (1 g, 13.88 mmol) was added dropwise to 36 mL ice cooled solution of ammonium hydroxide. The reaction mixture was stirred for 12 hours. The solvent was removed under vacuum and the residue was dissolved in methanol. Di-*tert*-butyl dicarbonate (7.5 g, 34.7 mmol) was added to the reaction mixture and stirred for 4 hours. The mixture was purified using column chromatography (24% EtOAc/hexane) to obtain *tert*-butyl 2-hydroxy-2-methylpropylcarbamate (2.1 g). The pure *tert*-butyl 2-hydroxy-2-methylpropylcarbamate was dissolved in 8 mL of trifluoroacetic acid and stirred for 35 minutes. The solvent was removed under reduced pressure to afford 1-amino-2-methylpropan-2-ol as colorless oil (2.13 g, 80%).

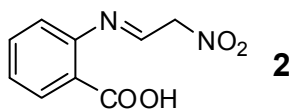
### Synthesis of Compound 20: methyl 2-(ethylamino)acetate.



To a solution of methyl 2-aminoacetate **19** (2 g, 22.5 mmol) in anhydrous methanol was added acetaldehyde (346 mg, 7.8 mmol), sodium cyanoborohydride (496 mg, 7.8 mmol) and 5-6 drops of acetic acid. The reaction mixture was stirred for 12 hours. Concentrated HCl was added to the reaction mixture till the pH became acidic (1-2). The solvent was removed under reduced pressure and the residue was purified using column chromatography (5% methanol/dichloromethane) to afford methyl 2-(ethylamino)acetate **20** as colorless oil (373 mg, 57%). <sup>1</sup>H NMR (500 MHz, MeOD) δ 3.90 (s, 2H), 3.75 (s, 3H), 3.03 (q, *J* = 7.3 Hz, 2H), 1.23 (t, *J* = 7.3 Hz, 3H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 168.28, 53.53, 47.75, 43.97, 11.35. MS (ESI) calculated for C<sub>5</sub>H<sub>11</sub>NO<sub>2</sub>, *m/z* 117.08, found 118.09 (*M* + H)<sup>+</sup>.

**Synthesis of Compound 22: 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid.**

Methyl 2-(ethylamino)acetate **20** (200 mg, 1.71 mmol) was dissolved in 10 mL of methanol. Di-*tert*-butyl dicarbonate (746 mg, 3.42 mmol) and triethylamine (207 mg, 2.05 mmol) was added to the reaction mixture. The reaction was monitored by thin layer chromatography, and after 2 hours the solvent was evaporated under vacuum. The residue was dissolved in 10 mL of THF/methanol (3:1). A solution of lithium hydroxide (164 mg, 6.84 mmol) in 2 mL of water was added to the reaction mixture. Reaction mixture was stirred for 12 hours and then solvent was removed under reduced pressure. Water was added to the residue, followed by acidification of the residue using 10% HCl until the pH dropped to ~2. The residue was then dissolved in ethylacetate, washed with water and dried over sodium sulfate to afford 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid **22** as colorless oil (274 mg, 79%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 4.00 (s, 1H), 3.91 (s, 1H), 3.34 (dq, *J* = 28.4, 6.9 Hz, 2H), 1.51 – 1.37 (m, 9H), 1.12 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 174.05, 173.22, 154.14, 152.91, 78.61, 78.42, 46.46, 46.40, 41.50, 40.80, 26.32, 26.21, 11.50, 11.19. Doubling of resonances due to tautomerization was observed. MS (ESI) calculated for C<sub>9</sub>H<sub>17</sub>NO<sub>4</sub>, *m/z* 203.12, found 202.11 (*M* - H)<sup>-</sup>.

**Synthesis of Compound 2: 2-(2-nitroethylideneamino)benzoic acid.**

Nitromethane (4.32 mL, 80 mmol) was added dropwise to a solution of NaOH (9.6 g, 250 mmol) in 10 mL of water, kept in an ice-bath. The mixture was then warmed to 40 °C and nitromethane (4.32 mL, 80 mmol) was again added slowly at 40-45 °C. The temperature was maintained until a clear solution was obtained. The reaction mixture was then heated to 50-55 °C for 2-5 minutes, cooled to

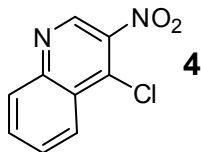
30 °C, poured onto ice and acidified with 22 mL conc. HCl. The resultant solution of methazonic acid was added immediately to a filtered solution of anthranilic acid **1** (10 g, 73 mmol) and 6.6 mL of conc. HCl in 75 mL of water. The reaction mixture was allowed to stand at room temperature for 12 hours. After filtration, the residue obtained was washed with water, and dried at 110 °C to yield a yellow solid powder of 2-(2-nitroethylideneamino)benzoic acid **2** (12.94 g, 85%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.14 (d, *J* = 7.7 Hz, 1H), 7.83 (d, *J* = 6.1 Hz, 1H), 7.68 – 7.59 (m, 2H), 7.22 (t, *J* = 7.4 Hz, 1H), 6.73 (d, *J* = 6.3 Hz, 1H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 168.57, 141.25, 136.64, 134.31, 131.98, 123.21, 116.75, 114.55, 99.99. MS (ESI) calculated for C<sub>9</sub>H<sub>8</sub>N<sub>2</sub>O<sub>4</sub>, *m/z* 208.05, found 207.04 (*M* - H)<sup>-</sup>.

### Synthesis of Compound 3: 3-nitroquinolin-4-ol.



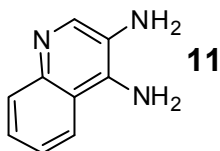
A solution of 2-(2-nitroethylideneamino)benzoic acid **2** (12.94 g, 62.2 mmol) in 50 mL of acetic anhydride was placed in a 2-neck flask fitted with a reflux condenser. It was stirred and heated to 100-105 °C until a clear solution was obtained. Heating was then discontinued and potassium acetate (6.22 g, 63.5 mmol) was added. The mixture was then again refluxed for 15 minutes with vigorous stirring, until a solid started to precipitate. The reaction mixture was then slowly cooled to room temperature. The residue was filtered, washed with glacial acetic acid until the washings were colorless, then suspended in water, filtered, washed with water and dried at 110 °C to get 3-nitroquinolin-4-ol **3** (4.68 g, 40%). <sup>1</sup>H NMR (500 MHz, DMSO) δ 13.04 (s, 1H), 9.21 (s, 1H), 8.25 (dd, *J* = 8.1, 1.1 Hz, 1H), 7.83 – 7.77 (m, 1H), 7.74 – 7.70 (m, 1H), 7.52 (ddd, *J* = 8.1, 7.1, 1.1 Hz, 1H). <sup>13</sup>C NMR (126 MHz, DMSO) δ 167.64, 142.46, 138.25, 133.22, 130.89, 128.04, 125.95, 125.83, 119.49. MS (ESI) calculated for C<sub>9</sub>H<sub>6</sub>N<sub>2</sub>O<sub>3</sub>, *m/z* 190.04, found 213.03 (*M* + Na<sup>+</sup>).

#### Synthesis of Compound 4: 4-chloro-3-nitroquinoline.



3-Nitroquinolin-4-ol **3** (4.5 g, 23.7 mmol) was added to 30 mL of phosphorous oxychloride with stirring. The reaction mixture was refluxed for 30 minutes. The solvent was then evaporated under vacuum and the residue was poured over crushed ice while stirring. After an hour the solid that formed was filtered, washed with cold water and dissolved in dichloromethane and some methanol. The solution was then extracted with ice cold NaOH (1 N) and dried over sodium sulfate, with the addition of activated charcoal. The solution was filtered over celite, and the solvent was evaporated. The residue was triturated with diethyl ether and dried under vacuum to obtain 4-chloro-3-nitroquinoline **4** (3.69 g, 75%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.28 (s, 1H), 8.50 – 8.44 (m, 1H), 8.24 (d,  $J$  = 8.5 Hz, 1H), 8.01 – 7.94 (m, 1H), 7.84 (t,  $J$  = 7.7 Hz, 1H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  149.23, 144.44, 141.23, 136.53, 133.10, 130.27, 129.66, 125.95, 125.51. MS (ESI) calculated for  $\text{C}_9\text{H}_4\text{ClN}_2\text{O}_2$ ,  $m/z$  208.00, found 209.01 ( $\text{M} + \text{H}$ ) $^+$ .

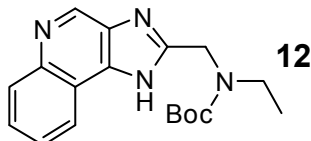
#### Synthesis of Compound 11: quinoline-3,4-diamine.



To a solution of 4-chloro-3-nitroquinoline **4** (1 g, 4.87 mmol) in 15 mL of DMF, sodium azide (625 mg, 9.62 mmol) was added. The reaction was stirred for an hour and solvent was evaporated under vacuum. The residue was dissolved in dichloromethane, washed with water, followed by drying over sodium sulfate. The filtrate was concentrated under reduced pressure to get crude 4-azido-3-nitroquinoline **10** (1 g, 97% crude). The crude **10** was then dissolved in methanol and subjected to catalytic hydrogenation over Pd/C at 60 psi hydrogen pressure for 4 hours. The solution was then filtered using celite and filtrate was evaporated under reduced pressure to afford the quinoline-3,4-

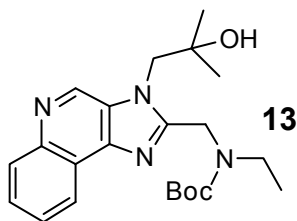
diamine **11** (700 mg, 95%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  8.22 (s, 1H), 7.96 (d,  $J$  = 8.4 Hz, 1H), 7.72 (d,  $J$  = 8.5 Hz, 1H), 7.43 (t,  $J$  = 7.5 Hz, 1H), 7.36 (t,  $J$  = 7.5 Hz, 1H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  141.66, 139.29, 136.20, 125.60, 124.41, 122.57, 122.13, 118.92, 116.93. MS (ESI) calculated for  $\text{C}_9\text{H}_9\text{N}_3$ ,  $m/z$  159.08, found 160.09 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 12:** *tert*-butyl (1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl) carbamate.



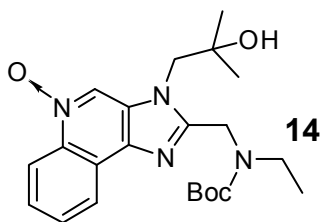
Quinoline-3,4-diamine **11** (161 mg, 1 mmol), 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid **22** (165 mg, 0.8 mmol), *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyl uronium hexafluorophosphate (HATU) (426 mg, 1.1 mmol), triethylamine (113 mg, 1.1 mmol) and catalytic amount of DMAP were dissolved in 10 mL of DMF. The reaction mixture was stirred for 10-12 hours. The solvent was evaporated under vacuum. The residue was dissolved in ethylacetate, washed with water and dried over sodium sulfate to obtain the crude residue. The crude residue was dissolved in 10 mL of ethanol and solution of NaOH (105 mg, 2.62 mmol) in 1 mL of water was added to it. The reaction mixture was refluxed for 5-6 hours, followed by evaporation under reduced pressure. The crude mixture was purified by column chromatography (3% methanol/dichloromethane) to obtain *tert*-butyl (1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate **12** (158 mg, 60%).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.98 (s, 1H), 8.31 (d,  $J$  = 6.0 Hz, 1H), 8.03 (d,  $J$  = 8.5 Hz, 1H), 7.66 – 7.55 (m, 2H), 4.71 (s, 2H), 3.39 (m, 2H), 1.42 (s, 5H), 1.20 (s, 4H), 1.08 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  175.53, 157.20, 152.53, 144.10, 129.79, 127.40, 126.60, 121.11, 81.10, 45.32, 43.67, 29.70, 28.41, 13.64. MS (ESI) calculated for  $\text{C}_{18}\text{H}_{22}\text{N}_4\text{O}_2$ ,  $m/z$  326.17, found 327.18 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 13:** *tert*-butyl ethyl((3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate.



To a solution of *tert*-butyl (1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate **12** (95 mg, 0.29 mmol) in 5 mL of 2,2-dimethyloxirane **17**, 1,8-Diazabicyclo[5.4.0]-undec-7-ene (DBU) (110 mg, 0.73 mmol) was added. The reaction mixture was refluxed for 18 hours. The solvent was evaporated under vacuum and the residue was purified using column chromatography (2% methanol/dichloromethane) to obtain *tert*-butyl ethyl((3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate **13** (80 mg, 70%). <sup>1</sup>H NMR (400 MHz, MeOD)  $\delta$  9.26 (s, 1H), 8.64 – 8.52 (m, 1H), 8.14 (dd, *J* = 6.6, 2.9 Hz, 1H), 7.77 – 7.67 (m, 2H), 4.95 (s, 2H), 4.52 (s, 2H), 3.48 (s, 2H), 1.51 (s, 6H), 1.32 (s, 9H), 1.22 – 1.13 (m, 3H). <sup>13</sup>C NMR (126 MHz, MeOD)  $\delta$  154.82, 153.28, 142.27, 141.88, 136.95, 128.72, 126.91, 126.29, 125.72, 120.63, 120.26, 79.27, 69.60, 53.15, 41.76, 40.86, 26.16, 25.12, 10.88. MS (ESI) calculated for C<sub>22</sub>H<sub>30</sub>N<sub>4</sub>O<sub>3</sub>, *m/z* 398.23, found 421.22 (*M* + Na<sup>+</sup>).

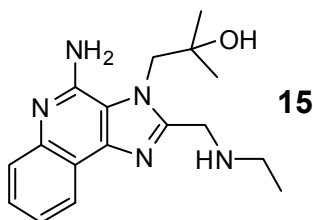
**Synthesis of Compound 14: 2-((*tert*-butoxycarbonyl(ethyl)amino)methyl)-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinoline 5-oxide.**



*tert*-Butyl ethyl((3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate **13** (64 mg, 0.81 mmol) was dissolved in 10 mL of dichloromethane/chloroform (1:1) and 0.5 mL of methanol. 3-Chloro-peroxybenzoic acid (69 mg, 0.4 mmol) was added and the reaction mixture was refluxed for 30 minutes. The reaction mixture was concentrated under reduced pressure and the residue was purified using column chromatography (5% methanol/dichloromethane) to obtain 2-((*tert*-butoxycarbonyl(ethyl)amino)methyl)-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinoline

5-oxide **14** (228 mg, 72%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.46 (s, 1H), 8.70 (s, 1H), 8.49 (s, 1H), 7.83 – 7.65 (m, 2H), 4.94 (s, 2H), 4.33 (s, 2H), 3.35 (s, 2H), 1.53 (s, 9H), 1.39 (s, 6H), 1.08 (t,  $J$  = 6.9 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  154.02, 151.88, 135.69, 135.52, 127.48, 126.90, 126.43, 120.53, 120.39, 118.18, 114.15, 78.87, 69.22, 53.46, 41.31, 39.47, 26.51, 25.16, 11.03. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_4$ ,  $m/z$  414.23, found 415.23 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 15: 1-(4-amino-2-((ethylamino)methyl)-3*H*-imidazo[4,5-*c*]quinolin-3-yl)-2-methylpropan-2-ol.**

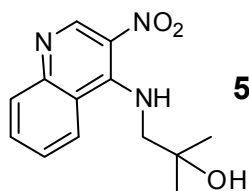


2-((*tert*-Butoxycarbonyl(ethyl)amino)methyl)-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinoline 5-oxide **14** (40 mg, 0.1 mmol) was dissolved in 4 mL of anhydrous dichloromethane. Benzoylisocyanate (29 mg, 0.19 mmol) was added to the reaction mixture and refluxed for 30 minutes. The solvent was then removed under vacuum and the residue was dissolved in 5 mL of anhydrous methanol. Excess of sodium methoxide was added and the reaction mixture was refluxed for 2-3 hours. After evaporating solvent under reduced pressure, the crude residue was purified using column chromatography (3% methanol/dichloromethane) to afford *tert*-butyl (4-amino-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate (30 mg, 75%). The *tert*-butyl (4-amino-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate (27 mg, 0.07 mmol) was dissolved in 5 mL trifluoroacetic acid and stirred for 35 minutes. The solvent was removed under reduced pressure to afford quantitatively 35 mg of 1-(4-amino-2-((ethylamino)methyl)-3*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol as trifluoroacetate salt **15**. The compound was eluted using column chromatography to obtain the free base form of **15** (10% methanol/dichloromethane).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  8.35 (d,  $J$  = 8.0 Hz, 1H), 7.69 (d,  $J$  = 8.3 Hz, 1H), 7.58 – 7.52 (m, 1H), 7.40 (t,  $J$  = 7.5 Hz, 1H), 4.55 (s, 2H), 4.37 (s, 2H), 3.07 – 2.96 (m, 2H),



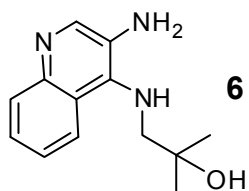
1.35 (s, 6H), 1.30 (t,  $J = 7.2$  Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  152.66, 148.34, 144.65, 141.87, 127.69, 123.62, 122.99, 121.05, 120.44, 118.80, 70.07, 54.56, 44.34, 43.23, 26.12, 12.17. MS (ESI) calculated for  $\text{C}_{17}\text{H}_{23}\text{N}_5\text{O}$ ,  $m/z$  313.19, found 314.20 ( $\text{M} + \text{H}$ ) $^+$ .

#### Synthesis of Compound 5: 2-methyl-1-(3-nitroquinolin-4-ylamino)propan-2-ol.



The trifluoroacetate salt of 1-amino-2-methylpropan-2-ol **18** (974 mg, 4.8 mmol) was added to the solution of 4-chloro-3-nitroquinoline **4** (500 mg, 2.4 mmol) and *N,N*-diethylpropan-2-amine (1.07 mL, 6 mmol) in 4:1 mixture of toluene and 2-propanol. The mixture was heated to 70 °C for half an hour until a solid started precipitating. The reaction mixture was then cooled, filtered, washed with toluene/2-propanol (7:3), ether and cold water. The residue was dried at 80 °C to obtain 2-methyl-1-(3-nitroquinolin-4-ylamino)propan-2-ol **5** (450 mg, 72%).  $^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  9.91 (s, 1H), 9.18 (s, 1H), 8.47 (d,  $J = 8.4$  Hz, 1H), 7.91 (d,  $J = 8.1$  Hz, 1H), 7.85 (t,  $J = 7.5$  Hz, 1H), 7.59 (t,  $J = 7.5$  Hz, 1H), 5.15 (s, 1H), 3.86 (d,  $J = 4.5$  Hz, 2H), 1.18 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz, DMSO)  $\delta$  151.14, 150.15, 147.31, 133.28, 129.92, 128.15, 126.08, 125.60, 119.55, 69.25, 59.48, 27.55. MS (ESI) calculated for  $\text{C}_{13}\text{H}_{15}\text{N}_3\text{O}_3$ ,  $m/z$  261.11, found 262.12 ( $\text{M} + \text{H}$ ) $^+$ .

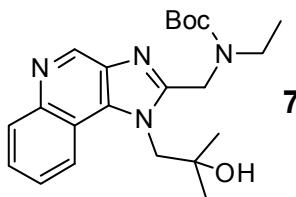
#### Synthesis of Compound 6: 1-(3-aminoquinolin-4-ylamino)-2-methylpropan-2-ol.



2-Methyl-1-(3-nitroquinolin-4-ylamino)propan-2-ol **5** (450 mg, 1.72 mmol) was dissolved in methanol and hydrogenated over Pd/C as catalyst at 60 psi hydrogen pressure for 4 hours. The solution was then filtered using celite, followed by evaporation of solvent under reduced pressure to afford 1-(3-

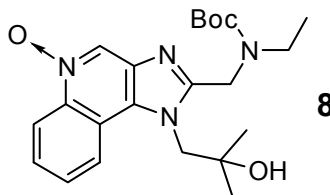
aminoquinolin-4-ylamino)-2-ethylpropan-2-ol **6** (380 mg, 96%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  8.37 (s, 1H), 8.11 – 8.04 (m, 1H), 7.85 – 7.78 (m, 1H), 7.50 – 7.43 (m, 2H), 3.34 (s, 2H), 1.29 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  142.97, 142.19, 137.67, 130.54, 127.43, 125.63, 125.22, 122.82, 120.93, 70.50, 56.39, 26.07. MS (ESI) calculated for  $\text{C}_{13}\text{H}_{17}\text{N}_3\text{O}$ ,  $m/z$  231.14, found 232.14 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 7: *tert*-butyl ethyl((1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl) carbamate.**



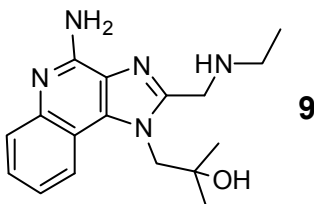
1-(3-Aminoquinolin-4-ylamino)-2-methylpropan-2-ol **6** (108 mg, 0.24 mmol), 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid **22** (43 mg, 0.21 mmol), *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyl uronium hexafluorophosphate (HATU) (109 mg, 0.29 mmol), triethylamine (73 mg, 0.72 mmol) and catalytic amount of DMAP were dissolved in 5 mL of DMF. The reaction mixture was stirred for 10-12 hours. The solvent was evaporated under vacuum and the residue was dissolved in ethylacetate, washed with water, dried over sodium sulfate and concentrated under reduced pressure to obtain the crude residue. The crude residue was dissolved in 10 mL of ethanol and solution of sodium hydroxide (33 mg, 0.83 mmol) in 1 mL of water was added. The reaction mixture was refluxed for 5-6 hours and the crude mixture was purified using column chromatography (4% methanol/dichloromethane) to obtain *tert*-butyl (1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate **7** (82 mg, 80%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.26 (s, 1H), 8.34 (d,  $J$  = 8.2 Hz, 1H), 8.24 (d,  $J$  = 8.3 Hz, 1H), 7.63 (dt,  $J$  = 15.3, 7.0 Hz, 2H), 4.72 (s, 4H), 3.69 (s, 1H), 3.32 (s, 2H), 1.52 (s, 9H), 1.45 – 1.12 (m, 6H), 1.03 (t,  $J$  = 7.0 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  152.39, 145.33, 145.15, 136.12, 135.19, 131.17, 126.92, 125.93, 120.37, 118.31, 80.82, 72.14, 55.43, 44.28, 41.68, 28.40, 12.87. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_3$ ,  $m/z$  398.23, found 399.17 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 8: 2-((*tert*-butoxycarbonyl(ethyl)amino)methyl)-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo [4,5-*c*]quinoline 5-oxide.**



*tert*-Butyl ethyl((1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate **7** (270 mg, 0.68 mmol) was dissolved in 20 mL of dichloromethane/chloroform (1:1) and 2 mL of methanol. 3-Chloro-peroxybenzoic acid (326 mg, 1.7 mmol) was added and the reaction mixture was refluxed for 30 minutes. The reaction mixture was concentrated under reduced pressure and the residue was purified using column chromatography (7% methanol/dichloromethane) to afford 2-((*tert*-butoxycarbonyl (ethyl)amino)methyl)-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinoline 5-oxide **8** (228 mg, 82%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.72 (d, *J* = 8.4 Hz, 1H), 8.36 (s, 1H), 8.23 (s, 1H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.54 (d, *J* = 7.6 Hz, 1H), 5.48 (s, 2H), 4.69 (d, *J* = 28.5 Hz, 2H), 4.37 (s, 1H), 3.27 (d, *J* = 68.9 Hz, 2H), 1.65 – 1.40 (m, 15H), 1.00 (t, *J* = 7.0 Hz, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 153.52, 151.85, 135.13, 132.33, 127.83, 127.54, 125.64, 125.57, 119.99, 118.15, 115.86, 78.24, 69.22, 53.31, 40.95, 38.97, 25.94, 25.07, 10.47. MS (ESI) calculated for C<sub>22</sub>H<sub>30</sub>N<sub>4</sub>O<sub>4</sub>, *m/z* 414.23, found 437.18 (*M* + Na<sup>+</sup>).

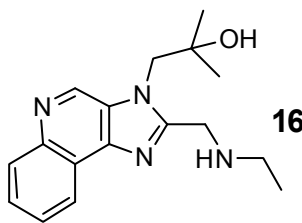
**Synthesis of Compound 9: 1-(4-amino-2-((ethylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methyl propan-2-ol.**



2-((*tert*-Butoxycarbonyl (ethyl)amino)methyl)-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinoline 5-oxide **8** (64 mg, 0.16 mmol) was dissolved in 5 mL of anhydrous dichloromethane.

Benzoylisocyanate (34 mg, 0.23 mmol) was added to the reaction mixture and refluxed for 30 minutes. The solvent was then removed under vacuum and the residue was dissolved in 5 mL of anhydrous methanol. Excess of sodium methoxide was added and the reaction mixture was refluxed for 2-3 hours. After evaporating solvent under reduced pressure, the crude residue was purified using column chromatography (7% methanol/dichloromethane) to obtain *tert*-butyl (4-amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate (46 mg, 72%). *tert*-Butyl (4-amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate (13 mg, 0.03 mmol) was dissolved in 3 mL of trifluoroacetic acid and stirred for 35 minutes. The solvent was removed under reduced pressure to afford quantitatively 16 mg of 1-(4-amino-2-((ethylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol as the trifluoroacetate salt **9**. The compound was eluted using column chromatography to obtain the free base form of **9** (13% methanol/dichloromethane). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.28 (d, *J* = 7.7 Hz, 1H), 7.73 (dd, *J* = 8.4, 1.0 Hz, 1H), 7.60 – 7.53 (m, 1H), 7.45 – 7.38 (m, 1H), 4.74 (s, 2H), 4.59 (s, 2H), 3.16 (q, *J* = 7.3 Hz, 2H), 1.37 (t, *J* = 7.3 Hz, 3H), 1.30 (s, 6H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 151.14, 149.51, 142.03, 135.36, 127.86, 125.74, 123.85, 122.53, 121.07, 114.71, 71.00, 55.27, 43.87, 42.97, 26.34, 11.26. MS (ESI) calculated for C<sub>17</sub>H<sub>23</sub>N<sub>5</sub>O, *m/z* 313.19, found 314.20 (*M* + H)<sup>+</sup>.

**Synthesis of Compound 16: 1-(2-((ethylamino)methyl)-3*H*-imidazo[4,5-*c*]quinolin-3-yl)-2-methylpropan-2-ol.**

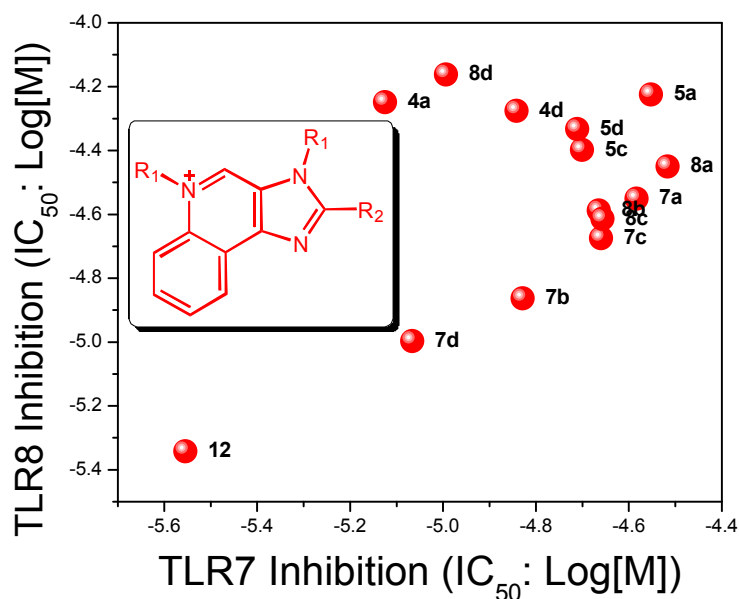


*tert*-Butyl ethyl((3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate **13** (15 mg, 0.04 mmol) was dissolved in 3 mL of trifluoroacetic acid and stirred for 35 minutes. The solvent was then removed under reduced pressure to obtain 1-(2-((ethylamino)methyl)-3*H*-imidazo[4,5-*c*]quinolin-3-yl)-2-methylpropan-2-ol as the trifluoroacetate salt **16** (19 mg, 95%). <sup>1</sup>H

NMR (500 MHz, MeOD)  $\delta$  9.62 (d,  $J$  = 20.0 Hz, 1H), 8.67 (d,  $J$  = 8.1 Hz, 1H), 8.17 (d,  $J$  = 8.3 Hz, 1H), 7.91 (dd,  $J$  = 11.3, 4.2 Hz, 1H), 7.87 (t,  $J$  = 7.5 Hz, 1H), 4.82 (s, 2H), 4.48 (s, 2H), 3.29 (q,  $J$  = 7.2 Hz, 2H), 1.36 (t,  $J$  = 7.3 Hz, 3H), 1.23 (s, 6H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  156.46, 148.73, 138.39, 135.72, 132.16, 131.12, 130.48, 123.81, 123.75, 122.85, 71.99, 56.31, 44.65, 44.63, 27.48, 11.48. MS (ESI) calculated for  $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}$ ,  $m/z$  298.18, found 299.19 ( $\text{M} + \text{H}$ ) $^+$ .

## Chapter 3.

# PRELIMINARY EVALUATION OF A 3*H* IMIDAZOQUINOLINE LIBRARY AS DUAL TLR7/TLR8 ANTAGONISTS



### 3.1. Introduction.

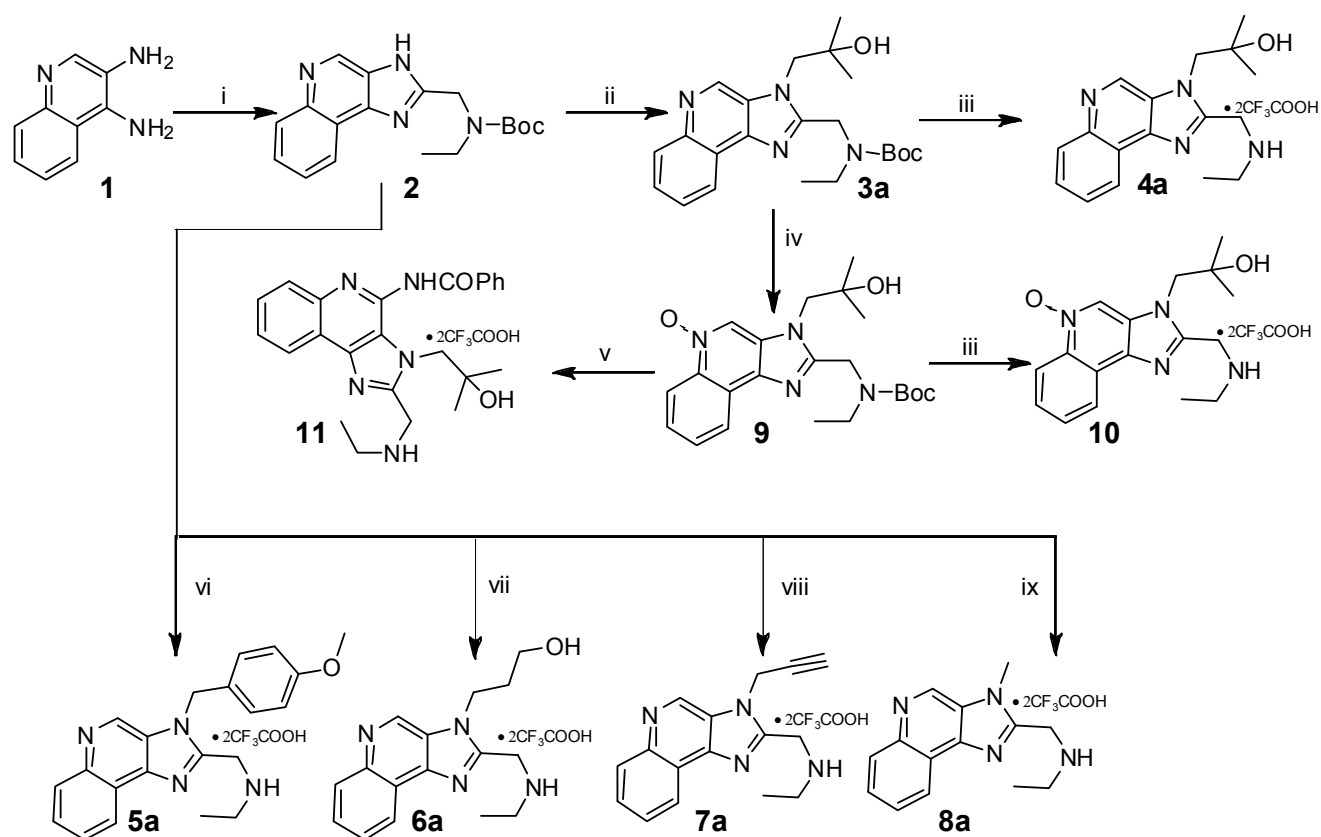
Chronic immune activation is a hallmark of several infectious and autoimmune diseases. Dysregulated cellular and humoral immune responses in progressive HIV infection,<sup>109;110</sup> for instance, leads to accelerated turnover of CD4<sup>+</sup> lymphocytes, thereby providing a milieu for HIV replication.<sup>111</sup> The engagement of toll-like receptor-7 (TLR7) by single-stranded viral RNA (ssRNA) has recently been reported to play a central role in immune activation-driven HIV replication.<sup>117</sup> Progressive CD4<sup>+</sup> T lymphocyte depletion in non-human primate models is highly correlated with TLR7-mediated interferon- $\alpha$  (IFN- $\alpha$ ) production by plasmacytoid dendritic cells, and antagonists of TLR7 have been shown to inhibit immune activation.<sup>117</sup> In contrast to the predominantly CD4<sup>+</sup> T lymphocyte-driven activation by TLR7- and 8- mediated recognition of HIV ssRNA, autoreactive B lymphocytes are thought to play an important role in the sustained generation of autoantibodies directed against both cytosolic and nuclear components contributing to the pathophysiology of disease states such as Systemic Lupus Erythematosus and Sjögren's syndrome.<sup>124-129</sup> TLR7 and TLR8 are thus logical targets for pharmacological intervention, and inhibitors for these endosomal receptors are being actively studied for potential use in the therapy of such autoimmune diseases.<sup>125;130;131</sup>

Whereas small molecule agonists of TLR7 are well known,<sup>69;119;132</sup> the only known class of TLR7 *antagonists*, until recently, were single-stranded phosphorothioate oligonucleotides.<sup>133-136</sup> En route to the synthesis of a TLR7-agonistic imidazoquinoline, gardiquimod, we synthesized its 3*H* regioisomer, which was found to be a weak TLR7 antagonist (Chapter 2).<sup>137</sup> A *des*-amino precursor of the 3*H* regioisomer (**4a**, Scheme 1) was more potent as a TLR7 antagonist, with an IC<sub>50</sub> value of 7.5  $\mu$ M;<sup>137</sup> negligible TLR8-antagonistic activity, however, was observed with this compound. Given the potential value of a detailed exploration of this chemotype toward obtaining leads for novel, and more potent TLR7/8 dual antagonists, we undertook the syntheses and evaluation of a preliminary library of 3*H* imidazoquinolines with the aim of identifying potential chemotypes capable of inhibiting both TLR7 and TLR8.

### 3.2. Results and Discussion.

Our point of departure was **4a** (Scheme 1), a 4-desamino, 3*H* imidazoquinoline with a 2-methylpropan-2-ol substituent at *N*<sup>3</sup>, and a 2-(ethylamino)methylene substituent at C2.<sup>137</sup> We first synthesized the *N*-oxide and C4-*N*-benzoyl derivatives (**10**, and **11**, respectively; Scheme 1). Upon finding that neither compound showed any appreciable activity (Table 1), we elected to first examine a small subset of compounds with varying substituents at *N*<sup>3</sup> (**5a**, **6a**, **7a**, **8a**; Scheme 1). These compounds also were found to be either inactive (**6a**) or of low potency (**5a**, **7a**, **8a**; Table 1).

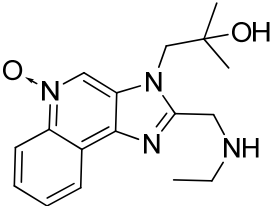
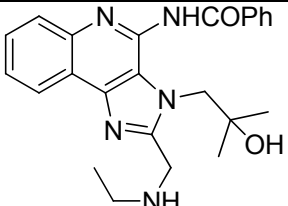
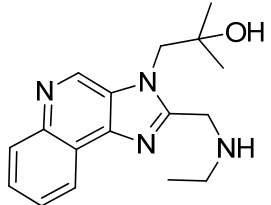
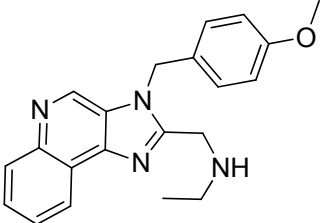
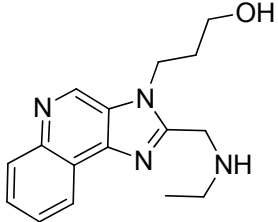
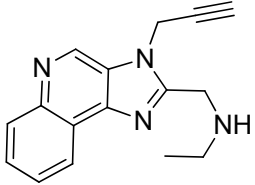
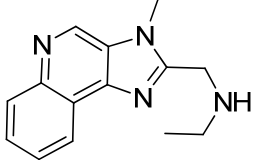
**Scheme 1.** Syntheses of derivatives of **4a** and *N*<sup>3</sup>-substituted 3*H* imidazoquinolines.



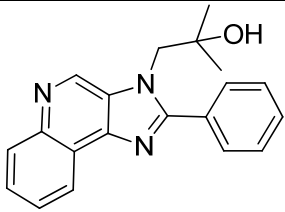
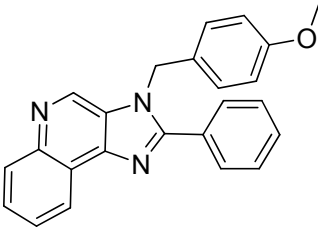
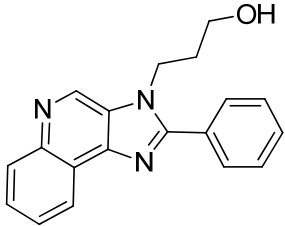
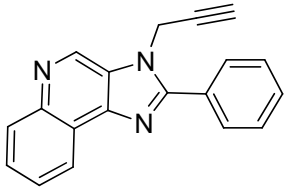
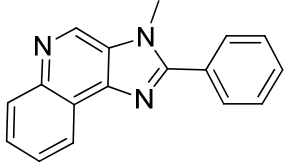
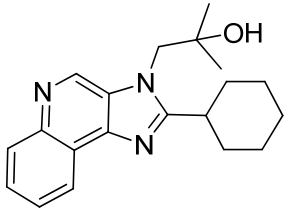
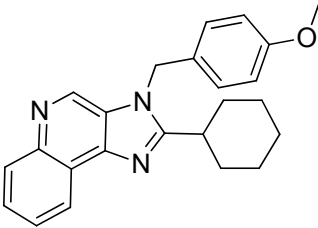
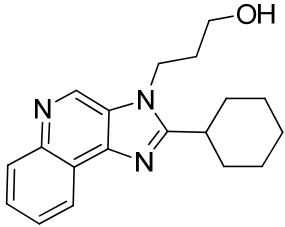
**Reagents:** i. 2-(*tert*-Butoxycarbonyl(ethyl)amino)acetic acid, HATU, DMF (b) NaOH/H<sub>2</sub>O, EtOH; ii. DBU, 2,2-dimethyloxirane; iii. CF<sub>3</sub>COOH; iv. 3-Chloroperoxybenzoic acid, CH<sub>2</sub>Cl<sub>2</sub>, CHCl<sub>3</sub>, MeOH; v. (a) Benzoyl isocyanate, CH<sub>2</sub>Cl<sub>2</sub> (b) CF<sub>3</sub>COOH. vi. 1-(Chloromethyl)-4-methoxybenzene, THF, 80 °C (b) CF<sub>3</sub>COOH; vii. 3-Bromo-1-propanol, DMF, 80 °C (b) CF<sub>3</sub>COOH; viii. Propargyl bromide, THF, 90 °C (b) CF<sub>3</sub>COOH; ix. Methyl iodide, DBU, THF (b) CF<sub>3</sub>COOH.



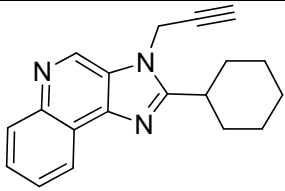
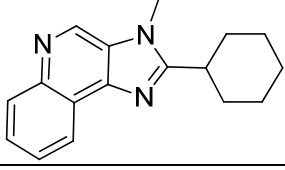
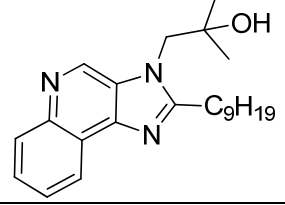
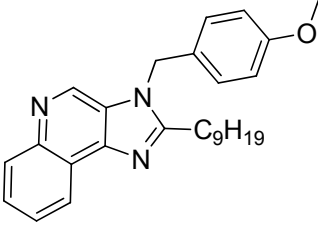
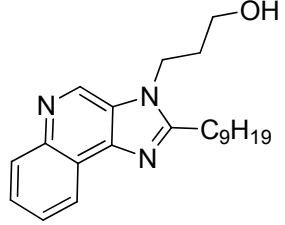
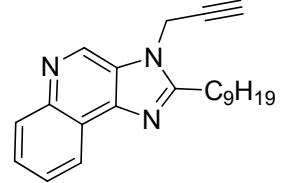
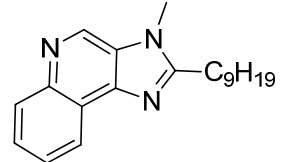
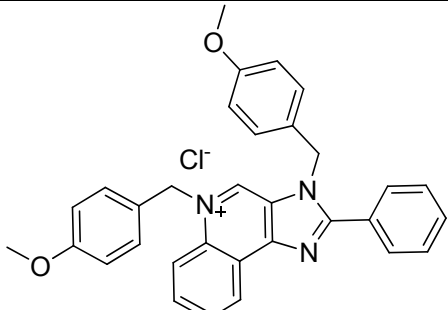
**Table 1.** TLR7- and TLR8-antagonistic activities of the title compounds. ND denotes no significant activity detected at the highest concentration tested (250  $\mu$ M).

Structure	Compound Number	TLR7 Antagonistic Activity ( $\mu$ M)	TLR8 Antagonistic Activity ( $\mu$ M)
	<b>10</b>	ND	46.5
	<b>11</b>	ND	21.17
	<b>4a</b>	7.5	56.31
	<b>5a</b>	28.02	59.64
	<b>6a</b>	ND	ND
	<b>7a</b>	26.1	28.13
	<b>8a</b>	30.44	35.43

**Table 1.** *contd.* N.D. denotes no significant activity detected at the highest concentration tested (250  $\mu$ M).

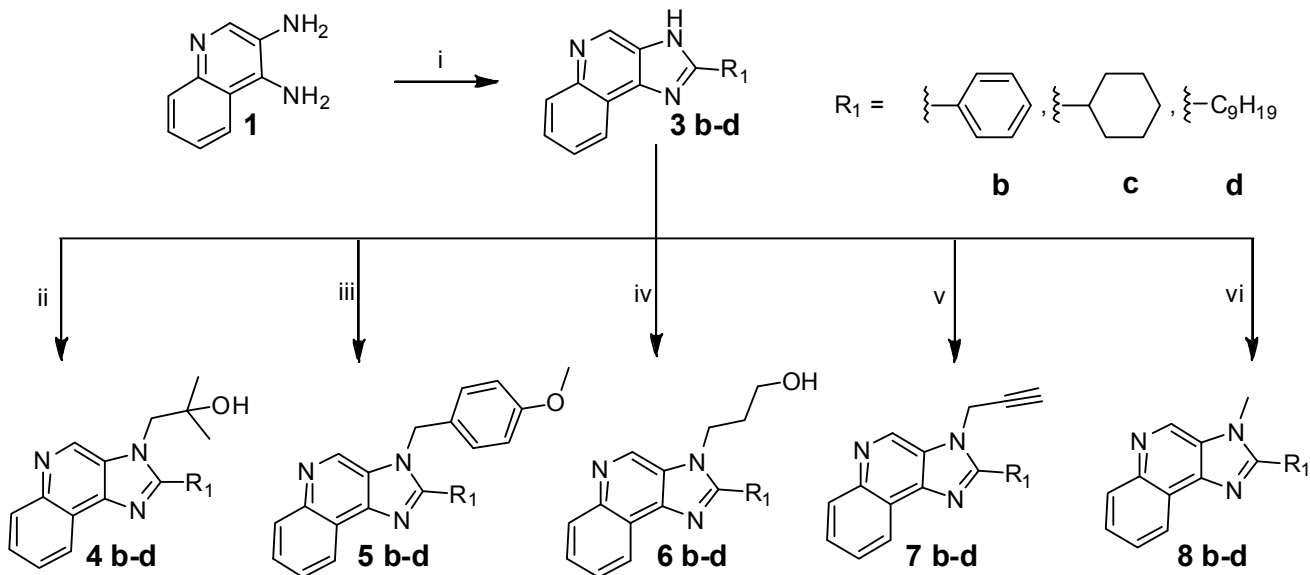
	<b>4b</b>	ND	38.42
	<b>5b</b>	ND	ND
	<b>6b</b>	ND	37.24
	<b>7b</b>	14.85	13.71
	<b>8b</b>	21.63	25.81
	<b>4c</b>	ND	38.73
	<b>5c</b>	19.9	40.01
	<b>6c</b>	ND	20.32

**Table 1.** contd. N.D. denotes no significant activity detected at the highest concentration tested (250  $\mu$ M).

	<b>7c</b>	21.87	21.12
	<b>8c</b>	22.04	24.3
	<b>4d</b>	14.4	52.94
	<b>5d</b>	19.44	46.4
	<b>6d</b>	ND	4.81
	<b>7d</b>	8.58	10.07
	<b>8d</b>	10.14	68.8
	<b>12</b>	2.79	4.55

We next attempted generating a small subset (15 compounds) in which the substituents at  $N^3$  and C2 were combinatorially varied. Since this was an exploratory library, we chose the C2 substituents from among planar, aromatic (phenyl), a cycloaliphatic (cyclohexyl), and a long-chain aliphatic (nonyl) groups (Scheme 2), while preserving the  $N^3$  substituents that we had used in Scheme 1. Alkylation of the **3** series of compounds afforded, as expected, three sets of regioisomers. In order to unambiguously characterize the position of the alkyl groups in these isomers, we correlated the crystal structure of **4b** with its 2D-NOESY spectrum (**Fig. 1**, overleaf). The NOESY spectrum showed diagnostic NOEs between the methylene protons on  $N^3$  (atom 20) with the quinoline proton (atom 9), as well as the phenyl protons (atoms 15, 19). Also seen, as would be expected, are NOEs between the methylene protons on  $N^3$  (atom 20) with the terminal dimethyl protons on the  $N^3$  substituent (**Fig. 1**).

**Scheme 2.** Syntheses of  $N^3$  and C2 modified 3H imidazoquinoline compounds.

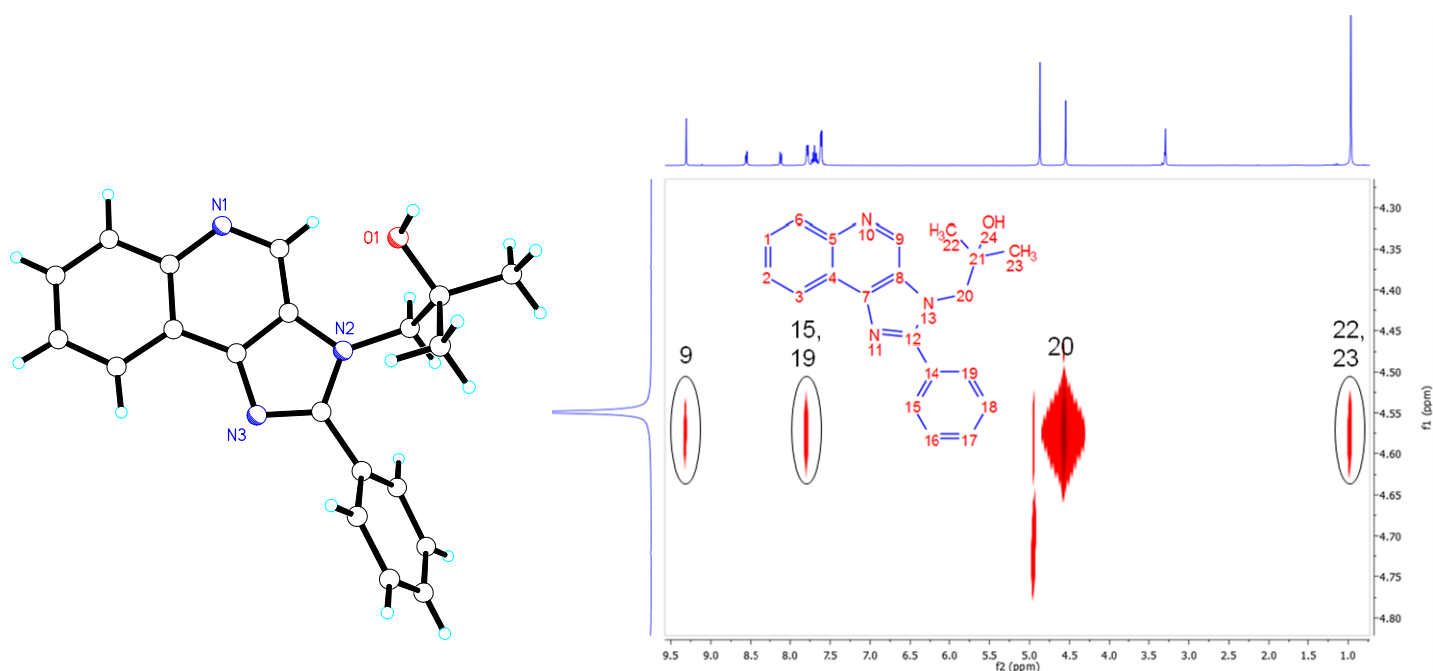


Reagents: i. Polyphosphoric acid,  $R_1$ -COOH, 180 °C; ii. DBU, 2,2-dimethyloxirane;  
 iii. 1-(Chloromethyl)-4-methoxybenzene, DBU, THF, 80 °C; iv. 3-Bromo-1-propanol, DBU, DMF, 80 °C;  
 v. Propargyl bromide, DBU, THF, 90 °C; vi. Methyl iodide, DBU, THF.

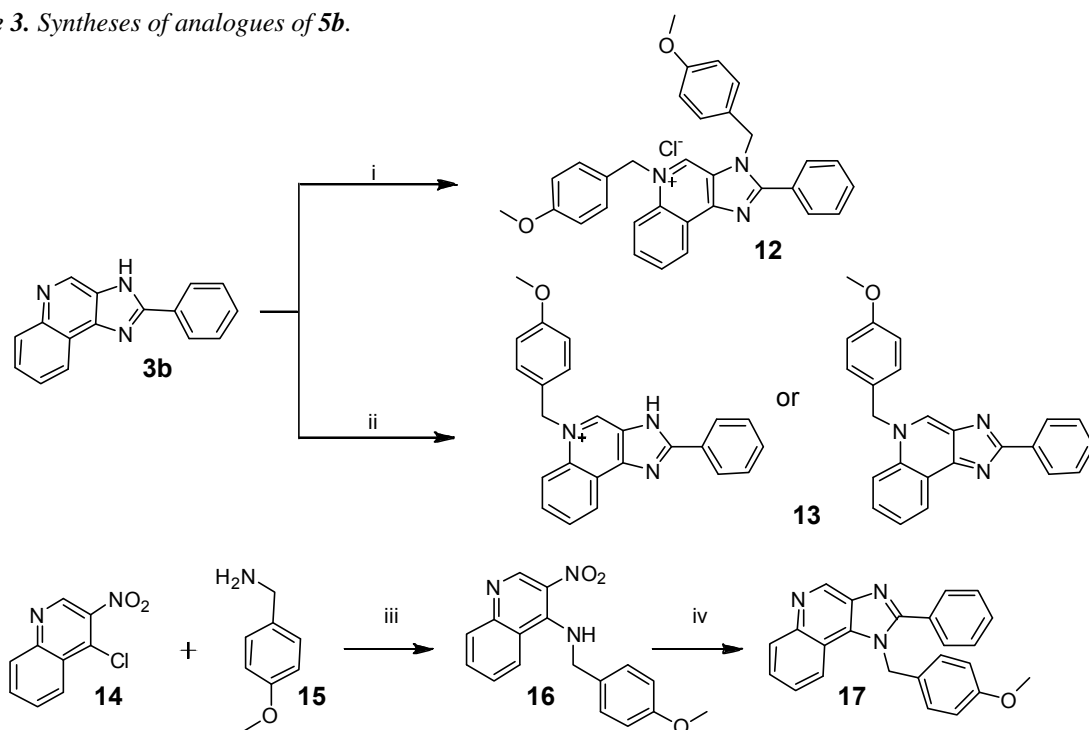
Most of these compounds displayed modest activity, with exceptions being the C2-nonyl-substituted **7d** and **8d** compounds exhibiting low micromolar TLR7-inhibitory activity. **7d** was also found to be TLR8-antagonistic (IC<sub>50</sub>: 10 μM, Table 1).

During the synthesis of **5b**, one of the side-products, **12**, corresponded in mass- and NMR-spectral characteristics to a *bis*-alkylated compound. This was isolated and was found to be the best-in-class in both TLR7- and TLR8-antagonism assays, with IC<sub>50</sub> values of 2.79 and 4.55 μM, respectively (Table 1). In order to determine whether **12** was a quinolinium or imidazolinium species, we synthesized the mono-alkylated quinolinium **13** and the 1*H* regioisomer **17** (Scheme 3, overleaf). The structure of compound **13** could be either the charged species or its uncharged tautomer as shown in Scheme 3. The dialkyl species **12** was obtained using an excess of 1-(chloromethyl)-4-methoxybenzene, using DBU as base, and in THF at 150 °C, whereas **13** was obtained in the absence of DBU and in DMF at 120°C. The 1*H* regioisomer **17** was synthesized by pre-installing the *p*-methoxybenzyl substituent on *N*<sup>1</sup> (Scheme 3). We characterized the monoalkylated regioisomers **5b**, **13**, and **17** (Scheme 3) via 2D-NOESY experiments (Fig. 2, overleaf).

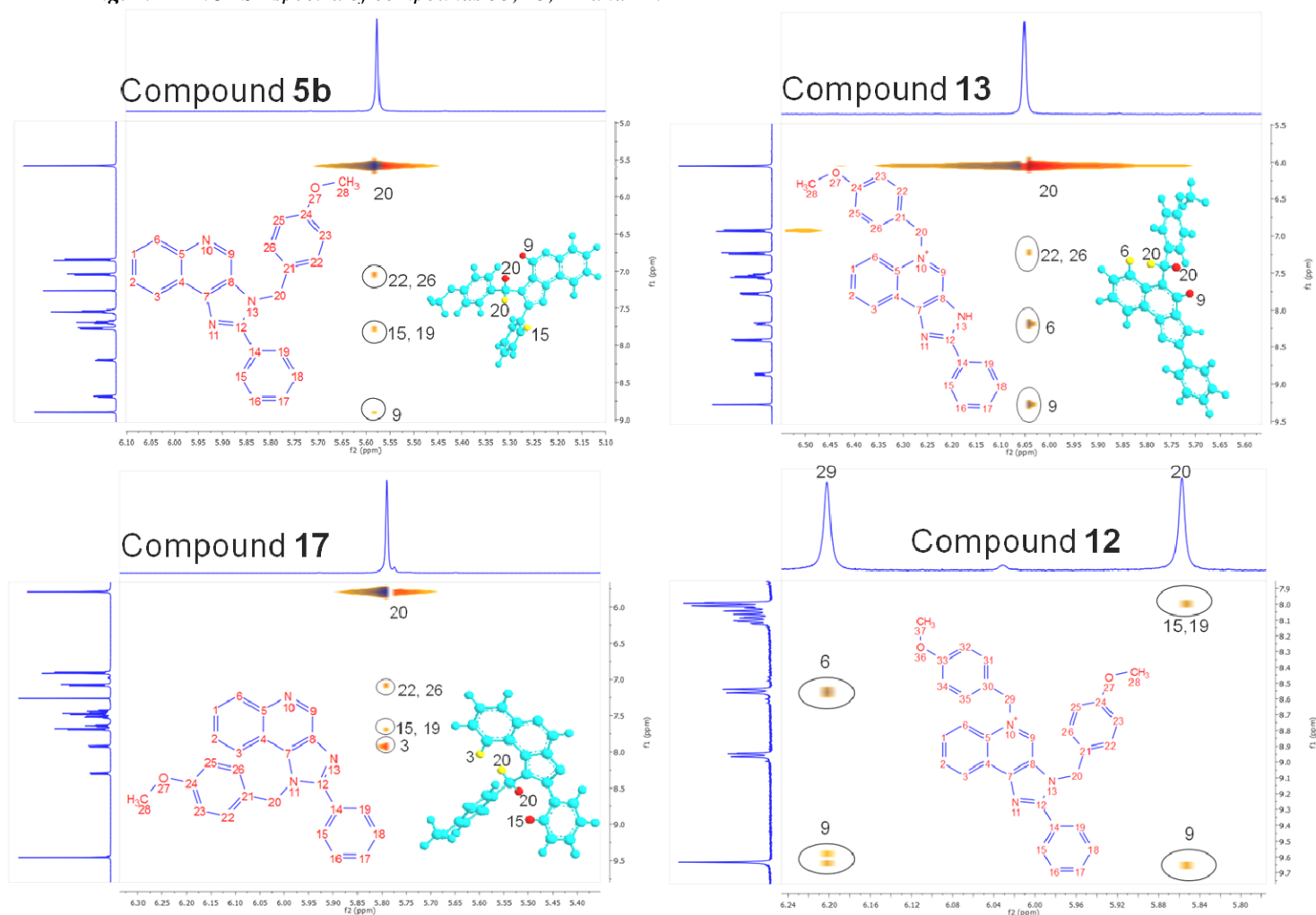
**Fig. 1.** Crystal structure (Left) and 2D-NOESY (right) of Compound **4b**.



**Scheme 3. Syntheses of analogues of 5b.**



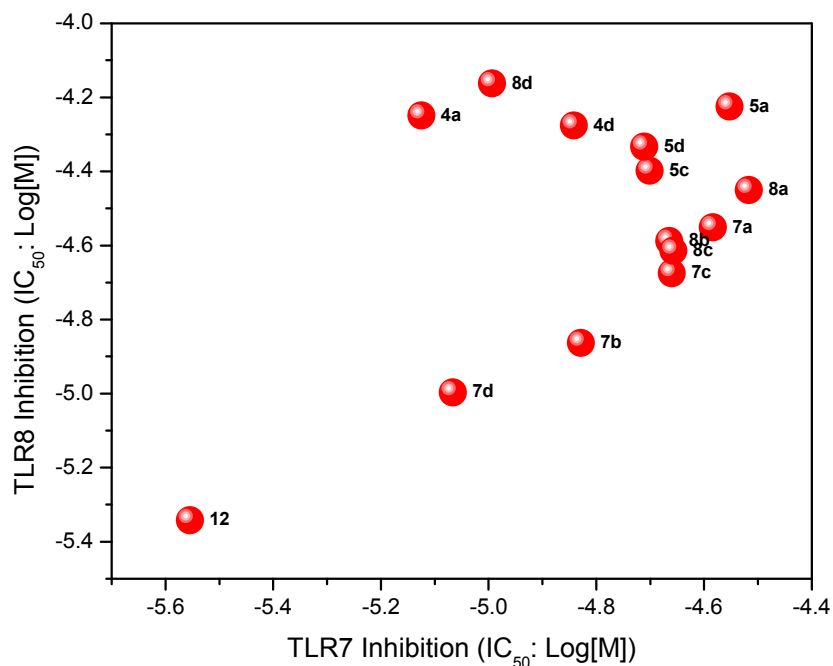
**Fig. 2. 2D-NOESY spectra of compounds 5b, 13, 17 and 12.**



Compounds **5b**, **13**, and **17** are all monoalkylated species, with the *p*-methoxybenzyl substituent at the  $N^3$  (imidazole),  $N^5$  (quinolinium),  $N^1$  (imidazole), respectively. Each of these compounds showed characteristic and diagnostic NOE crosspeaks. In **5b** (as in **4b**, discussed earlier), NOEs between the methylene protons on  $N^3$  (atom 20) with the quinoline proton (atom 9), as well as the phenyl protons (atoms 15, 19); compound **13** showed NOEs between the methylene protons on  $N^5$  (atom 20) with the quinoline protons (atoms 9 and 6); compound **17** was distinguished by crosspeaks between phenyl protons (atoms 15, 19) and the quinoline proton (atom 3) (**Fig. 2**). The clear NOE patterns helped establish unequivocally that the additional *p*-methoxybenzyl substituent in compound **12** was on the quinoline nitrogen ( $N^5$ ) because of NOEs similar to both **13** and **5b** (**Fig. 2**).

Compound **12**, to our considerable surprise, showed the highest potency in simultaneously inhibiting both TLR7 and TLR8 (Table 1, **Fig. 3**). This was unexpected since the quinolinium compound with its fixed charge is generally thought to be relatively membrane-impermeant and, as discussed earlier, both TLR7 and TLR8 are compartmentalized in the endosome.

**Fig. 3.** 2D-Scatter plot of TLR7 (abscissa) and TLR8 (ordinate) -antagonistic activities of the title compounds. Compounds that do not show significant inhibitory activity have been omitted.



### 3.3. Conclusions.

A pilot library of 3*H* imidazoquinolines have been synthesized, characterized, and evaluated for biological activity. Although possessing modest activity, a dual TLR7/TLR8 antagonist (**12**) has been identified with micromolar potencies. These preliminary results have been instructive in that they already point to strategies for improvement in potency. For instance, the monoalkylated compounds **7b** and **7d**, bearing propargyl groups on  $N^8$ , ranked next in potency to **12**, are attractive leads for additional alkylation with electron-rich substituents on the quinoline  $N^5$ . As mentioned earlier, the quaternary amine on the quinolinium of **12** may deter optimal trans-membrane transport and concentration in the endosomal compartment, and carbocyclic analogues may be evaluated to carefully examine the effect of the fixed charge.

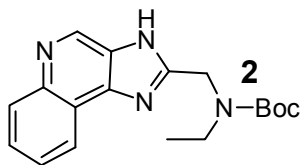
The principal thrust in our laboratory, however, is on evaluating TLR agonists as potential vaccine adjuvants. Future chapters (Chapters 4-7), consequently, focuses on the syntheses and evaluation of TLR7 and TLR8 agonistic 1*H* imidazoquinolines.



### 3.4. Experimental.

All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm x 150 mm, 5 µm analytical reverse phase C<sub>18</sub> column with H<sub>2</sub>O-isopropanol or H<sub>2</sub>O-CH<sub>3</sub>CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion acquisition mode. All the compounds synthesized were obtained as solids.

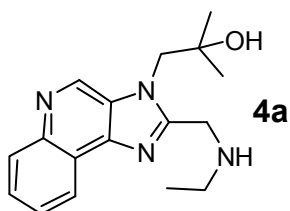
#### Synthesis of Compound 2: *tert*-butyl (1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl) carbamate.



To a solution of 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid (575 mg, 2.83 mmol) in anhydrous DMF, were added HATU (1.31 g, 3.46 mmol), triethylamine (350 mg, 3.46 mmol), a catalytic amount of DMAP and **1** (500 mg, 3.14 mmol). The reaction mixture was stirred for 10-12 hours. The solvent was then removed under vacuum and the residue was dissolved in EtOAc, washed with water, dried over sodium sulfate and concentrated to obtain the residue, which was dissolved in 30 mL of ethanol and a solution of NaOH (300 mg, 7.46 mmol) in 2 mL of water was added to it. The reaction mixture was refluxed for 5-6 hours, followed by evaporation under reduced pressure to obtain the residue, which was then purified using column chromatography (3% MeOH/dichloromethane) to obtain the compound **2** (575 mg, 56%). <sup>1</sup>H NMR (500 MHz, MeOD) δ 8.98 (s, 1H), 8.31 (d, *J* = 6.0 Hz, 1H), 8.03 (d, *J* = 8.5 Hz, 1H), 7.66 – 7.55 (m, 2H), 4.71 (s, 2H), 3.39 (d, *J* = 39.4 Hz, 2H), 1.42 (s, 5H), 1.20 (s,

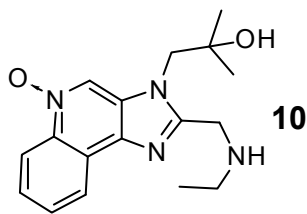
4H), 1.08 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  175.53, 157.20, 152.53, 144.10, 129.79, 127.40, 126.60, 121.11, 81.10, 45.32, 43.67, 29.70, 28.41, 13.64. MS (ESI) calculated for  $\text{C}_{18}\text{H}_{22}\text{N}_4\text{O}_2$ ,  $m/z$  326.1743, found 327.1209 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 4a: 1-(2-((ethylamino)methyl)-3*H*-imidazo[4,5-*c*]quinolin-3-yl)-2-methylpropan-2-ol.**



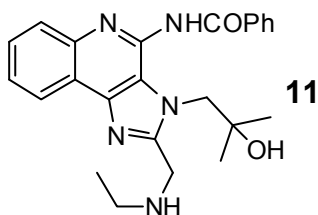
To a solution of **2** (95 mg, 0.29 mmol) in 5 mL of 2,2-dimethyloxirane, was added DBU (110 mg, 0.73 mmol). The reaction mixture was refluxed for 18 hours. The solvent was then evaporated under vacuum and the residue was purified using column chromatography (2% MeOH/dichloromethane) to obtain the compound **3a**. Compound **3a** (15 mg, 0.04 mmol) was then dissolved in 3 mL of trifluoroacetic acid and stirred for 35 minutes. The solvent was then removed under reduced pressure to obtain the compound **4a** (19 mg, 95%).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  9.62 (d,  $J$  = 20.0 Hz, 1H), 8.67 (d,  $J$  = 8.1 Hz, 1H), 8.17 (d,  $J$  = 8.3 Hz, 1H), 7.91 (dd,  $J$  = 11.3, 4.2 Hz, 1H), 7.87 (t,  $J$  = 7.5 Hz, 1H), 4.82 (s, 2H), 4.48 (s, 2H), 3.29 (q,  $J$  = 7.2 Hz, 2H), 1.36 (t,  $J$  = 7.3 Hz, 3H), 1.23 (s, 6H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  156.46, 148.73, 138.39, 135.72, 132.16, 131.12, 130.48, 123.81, 123.75, 122.85, 71.99, 56.31, 44.65, 44.63, 27.48, 11.48. MS (ESI) calculated for  $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}$ ,  $m/z$  298.1794, found 299.1860 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 10: 2-((ethylamino)methyl)-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinoline 5-oxide.**



Compound **3a** (145 mg, 0.36 mmol) was dissolved in 10 mL of anhydrous dichloromethane/chloroform (1:1) and 1 mL of anhydrous MeOH. 3-Chloro-peroxybenzoic acid (188 mg, 1.09 mmol) was added and the reaction mixture was refluxed for 30 minutes. The solvent was then removed under vacuum and the residue was purified using column chromatography (4% MeOH/dichloromethane) to obtain the compound **9** (100 mg, 66%). Compound **9** (28 mg, 0.07 mmol) was then dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes. The solvent was then removed and the residue was washed with diethyl ether to obtain the compound **10** (40 mg, 95%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  9.46 (s, 1H), 8.77 – 8.67 (m, 1H), 8.66 – 8.55 (m, 1H), 8.00 – 7.86 (m, 2H), 4.81 (s, 2H), 4.41 (s, 2H), 3.39 (q,  $J$  = 7.3, 2H), 1.48 (t,  $J$  = 7.3, 3H), 1.31 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  151.64, 139.07, 137.10, 129.59, 129.53, 128.75, 127.64, 122.22, 122.06, 119.15, 70.58, 54.56, 43.12, 43.07, 26.06, 10.09. MS (ESI) calculated for  $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}_2$ ,  $m/z$  314.1743, found 315.1764 ( $\text{M} + \text{H}$ ) $^+$ .

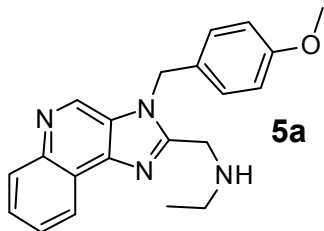
**Synthesis of Compound 11: *N*-(2-((ethylamino)methyl)-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-4-yl)benzamide.**



To a solution of **9** (68 mg, 0.16 mmol) in 5 mL of anhydrous dichloromethane, was added benzoylisocyanate (36 mg, 0.25 mmol) and the reaction mixture was refluxed for 30 minutes. The solvent was then removed under vacuum and the residue was purified using column chromatography to obtain *tert*-butyl (4-benzamido-3-(2-hydroxy-2-methylpropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl(ethyl)carbamate (30 mg, 35%), which was *N*-Boc deprotected by stirring in 5 mL of

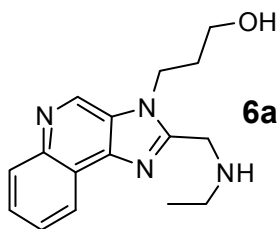
trifluoroacetic acid for 30 minutes. The solvent was then removed and the residue was washed with diethyl ether to obtain the compound **11** (35 mg, 95%).  $^1\text{H}$  NMR at 50°C (400 MHz, MeOD)  $\delta$  8.42 (d,  $J$  = 8.1 Hz, 1H), 8.25 (d,  $J$  = 7.3 Hz, 2H), 7.81 – 7.73 (m, 1H), 7.72 – 7.65 (m, 1H), 7.57 (t,  $J$  = 6.7 Hz, 2H), 7.50 (t,  $J$  = 7.4 Hz, 2H), 5.19 (s, 2H), 4.80 (s, 2H), 3.35 (q,  $J$  = 7.3 Hz, 2H), 1.45 (t,  $J$  = 7.3 Hz, 3H), 1.31 (s, 6H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  178.99, 155.88, 151.18, 145.60, 137.98, 133.28, 131.79, 129.13, 128.93, 128.15, 125.10, 122.17, 118.43, 117.99, 70.60, 56.37, 45.68, 44.04, 27.42, 14.81. MS (ESI) calculated for  $\text{C}_{24}\text{H}_{27}\text{N}_5\text{O}_2$ ,  $m/z$  417.2165, found 418.2137 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 5a:** *N*-((3-(4-methoxybenzyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)ethanamine.



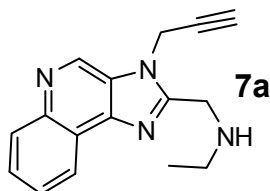
To a solution of **2** (50 mg, 0.15 mmol) in 1 mL of anhydrous THF, were added DBU (47 mg, 0.31 mmol) and 1-(chloromethyl)-4-methoxybenzene (96 mg, 0.61 mmol). The solution was then heated in a sealed vessel at 80 °C for 1 hour. After cooling to room temperature, and removing solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (1% MeOH/dichloromethane) to obtain the compound *tert*-butyl ethyl((3-(4-methoxybenzyl)-3*H*-imidazo [4,5-*c*]quinolin-2-yl)methyl)carbamate, which was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes. The solvent was then removed and the residue was washed with diethyl ether to obtain the compound **5a** (18 mg, 21%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  9.46 (s, 1H), 8.85 – 8.71 (m, 1H), 8.28 (d,  $J$  = 8.0, 1H), 8.07 – 7.93 (m, 2H), 7.31 (d,  $J$  = 8.8 Hz, 2H), 7.04 – 6.95 (m, 2H), 5.77 (s, 2H), 4.81 (s, 2H), 3.80 (s, 3H), 3.40 (q,  $J$  = 7.3 Hz, 2H), 1.48 (t,  $J$  = 7.3 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  160.28, 153.97, 147.68, 133.33, 130.79, 129.07, 128.77, 128.59, 125.90, 122.61, 122.29, 121.52, 114.47, 54.42, 47.95, 43.33, 42.91, 10.00. MS (ESI) calculated for  $\text{C}_{21}\text{H}_{22}\text{N}_4\text{O}$ ,  $m/z$  346.1794, found 347.1890 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 6a: 3-(2-((ethylamino)methyl)-3*H*-imidazo[4,5-*c*]quinolin-3-yl)propan-1-ol.**



To a solution of **2** (50 mg, 0.15 mmol) in 1 mL of anhydrous THF, were added DBU (47 mg, 0.31 mmol) and 3-bromopropan-1-ol (32 mg, 0.23 mmol). The solution was then heated in a sealed vessel at 80 °C for an hour. After cooling to room temperature, and removing solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography to obtain *tert*-butyl ethyl((3-(3-hydroxypropyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate (9 mg, 15%), which was dissolved in 8 mL of trifluoroacetic acid and stirred for 30 minutes. The solvent was then removed and the residue was washed with diethyl ether to obtain compound **6a** (11 mg, 95%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.24 (s, 1H), 8.60 – 8.52 (m, 1H), 8.21 – 8.09 (m, 1H), 7.81 – 7.67 (m, 2H), 4.68 (t, *J* = 6.8 Hz, 2H), 4.31 (s, 2H), 3.58 – 3.50 (m, 2H), 2.88 (q, *J* = 7.2 Hz, 2H), 2.28 – 2.14 (m, 2H), 1.25 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 154.25, 143.61, 136.25, 128.49, 128.17, 127.45, 126.88, 121.74, 121.20, 57.03, 44.36, 43.42, 40.91, 32.25, 13.04. MS (ESI) calculated for C<sub>16</sub>H<sub>20</sub>N<sub>4</sub>O, *m/z* 284.1637, found 285.1752 (*M* + *H*)<sup>+</sup>.

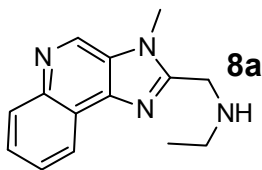
**Synthesis of Compound 7a: *N*-((3-(prop-2-ynyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)ethanamine.**



To a solution **2** (65 mg, 0.2 mmol) in 1 mL of anhydrous THF, were added DBU (61 mg, 0.4 mmol) and 80% propargyl bromide in toluene (119 mg, 1.0 mmol). The solution was then heated in a sealed

vessel at 90 °C for 30 minutes. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography to obtain *tert*-butyl ethyl((3-(prop-2-ynyl)-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate, which was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes. The solvent was then removed and the residue was washed with diethyl ether to obtain the compound **7a** (23 mg, 43%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.74 (s, 1H), 8.77 (dd, *J* = 8.1, 1.1 Hz, 1H), 8.30 (d, *J* = 8.1 Hz, 1H), 8.08 – 7.92 (m, 2H), 5.54 (d, *J* = 2.6 Hz, 2H), 4.95 (s, 2H), 3.45 (q, *J* = 7.2 Hz, 2H), 3.27 (t, *J* = 2.5 Hz, 1H), 1.52 (t, *J* = 7.3 Hz, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 153.17, 147.34, 137.95, 133.55, 130.69, 129.01, 128.14, 123.11, 122.21, 121.49, 76.40, 75.07, 43.38, 42.62, 34.26, 10.02. MS (ESI) calculated for C<sub>16</sub>H<sub>16</sub>N<sub>4</sub>, *m/z* 264.1375, found 265.1553 (*M* + *H*)<sup>+</sup>.

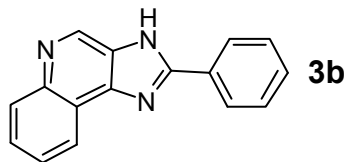
**Synthesis of Compound 8a: *N*-((3-methyl-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)ethanamine.**



To a solution of **2** (50 mg, 0.15 mmol) in 1 mL of anhydrous THF, were added DBU (47 mg, 0.31 mmol) and iodomethane (33 mg, 0.23 mmol). The solution was stirred for an hour and the solvent was then removed under vacuum. The residue was dissolved in EtOAc, washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (30% EtOAc/dichloromethane) to obtain *tert*-butyl ethyl((3-methyl-3*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)carbamate (7 mg, 20%), which was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes. The solvent was then removed and the residue was washed with diethyl ether to obtain the compound **8a** (13 mg, 95%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.72 (s, 1H), 8.79 (d, *J* = 7.9 Hz, 1H), 8.30 (d, *J* = 8.2 Hz, 1H), 8.14 – 7.91 (m, 2H), 4.91 (s, 2H), 4.18 (s, 3H), 3.45 (q, *J* = 7.3 Hz, 2H), 1.52 (t, *J* = 7.3 Hz, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 154.89, 147.95, 136.50, 132.91, 130.97, 129.23,

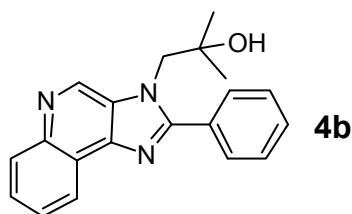
129.14, 122.38, 121.87, 121.36, 43.38, 42.48, 30.57, 10.01. MS (ESI) calculated for  $C_{14}H_{16}N_4$ ,  $m/z$  240.1375, found 241.1476 ( $M + H$ )<sup>+</sup>.

**Synthesis of Compound 3b: 2-phenyl-3*H*-imidazo[4,5-*c*]quinoline.**



To a mixture of **1** (450 mg, 2.83 mmol) and benzoic acid (691 mg, 5.66 mmol), was added polyphosphoric acid (appx. 25 mL) and heated to 180 °C for an hour. The reaction mixture was then slowly cooled to room temperature and the polyphosphoric acid was slowly neutralized with ammonium hydroxide until the pH was around 8. The compound was then extracted using EtOAc and the EtOAc fraction was then washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (4% MeOH/ dichloromethane) to afford the compound **3b** (315 mg, 45%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.07 (s, 1H), 8.44 (d,  $J$  = 6.9 Hz, 1H), 8.18 – 8.16 (m, 1H), 8.16 – 8.15 (m, 1H), 8.11 – 8.06 (m, 1H), 7.72 – 7.62 (m, 2H), 7.60 – 7.52 (m, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 143.22, 130.45, 129.06, 128.84, 128.14, 127.48, 126.74, 126.63, 121.39. MS (ESI) calculated for  $C_{16}H_{11}N_3$ ,  $m/z$  245.0953, found 246.1025 ( $M + H$ )<sup>+</sup>.

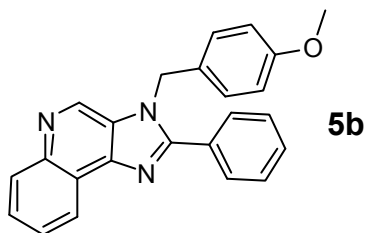
**Synthesis of Compound 4b: 2-methyl-1-(2-phenyl-3*H*-imidazo[4,5-*c*]quinolin-3-yl)propan-2-ol.**



To a solution of **3b** (50 mg, 0.2 mmol) in 1 mL of 2,2-dimethyloxirane, was added DBU (62 mg, 0.41 mmol) and the solution was heated under microwave conditions (600 W, 80 °C, 1h). After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and

washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (4% MeOH/dichloromethane) to obtain the compound **4b** (24 mg, 38 %).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  9.34 (s, 1H), 8.65 – 8.51 (m, 1H), 8.17 – 8.13 (m, 1H), 7.87 – 7.79 (m, 2H), 7.78 – 7.69 (m, 2H), 7.67 – 7.63 (m, 3H), 4.59 (s, 2H), 1.00 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  155.90, 143.40, 143.02, 139.02, 130.15, 129.95, 129.85, 129.76, 128.72, 128.04, 127.46, 126.80, 121.65, 121.37, 70.71, 55.09, 26.19. MS (ESI) calculated for  $\text{C}_{20}\text{H}_{19}\text{N}_3\text{O}$ ,  $m/z$  317.1528, found 318.1631 ( $\text{M} + \text{H}$ ) $^+$ .

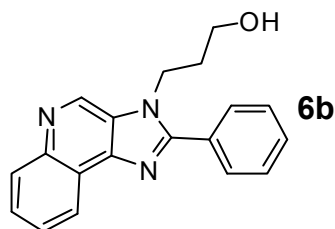
**Synthesis of Compound 5b: 3-(4-methoxybenzyl)-2-phenyl-3*H*-imidazo[4,5-*c*]quinoline.**



To a solution of **3b** (50 mg, 0.2 mmol) in 1 mL of anhydrous THF, were added DBU (62 mg, 0.41 mmol) and 1-(chloromethyl)-4-methoxybenzene (128 mg, 0.82 mmol). The solution was then heated in a sealed vessel at 80 °C for an hour. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (8% EtOAc/dichloromethane) to obtain the compound **5b** (36 mg, 33%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.92 (s, 1H), 8.73 – 8.69 (m, 1H), 8.24 – 8.20 (m, 1H), 7.82 – 7.77 (m, 2H), 7.74 – 7.69 (m, 2H), 7.59 – 7.53 (m, 3H), 7.06 (d,  $J$  = 8.8 Hz, 2H), 6.90 – 6.84 (m, 2H), 5.60 (s, 2H), 3.80 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  159.52, 154.86, 144.69, 144.54, 136.63, 130.42, 129.67, 129.62, 129.55, 129.11, 129.01, 128.30, 127.66, 127.53, 127.45, 126.86, 122.42, 121.87, 114.64, 55.33, 48.60. MS (ESI) calculated for  $\text{C}_{24}\text{H}_{19}\text{N}_3\text{O}$ ,  $m/z$  365.1528, found 366.1491 ( $\text{M} + \text{H}$ ) $^+$ .

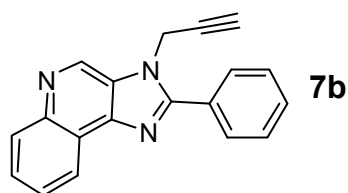


### Synthesis of Compound 6b: 3-(2-phenyl-3*H*-imidazo[4,5-*c*]quinolin-3-yl)propan-1-ol.



To a solution of **3b** (60 mg, 0.25 mmol) in 2 mL of anhydrous DMF, were added DBU (62 mg, 0.41 mmol) and 3-bromopropan-1-ol (139 mg, 1.0 mmol). The solution was then heated in a sealed vessel at 80 °C for 2 hours. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (20% acetone/dichloromethane) to obtain the compound **6b** (13 mg, 18%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.30 (s, 1H), 8.60 (dt, *J* = 5.0, 2.2 Hz, 1H), 8.21 – 8.17 (m, 1H), 7.89 – 7.85 (m, 2H), 7.79 – 7.71 (m, 2H), 7.67 (dd, *J* = 4.2, 2.4 Hz, 3H), 4.74 – 4.60 (m, 2H), 3.55 (t, *J* = 4.6 Hz, 2H), 2.12 – 2.03 (m, 2H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 155.37, 143.79, 143.53, 136.59, 130.46, 129.65, 129.38, 129.15, 128.78, 128.22, 127.59, 126.93, 121.70, 121.40, 58.02, 42.26, 32.70. MS (ESI) calculated for C<sub>19</sub>H<sub>17</sub>N<sub>3</sub>O, *m/z* 303.1372, found 304.1440 (*M* + *H*)<sup>+</sup>.

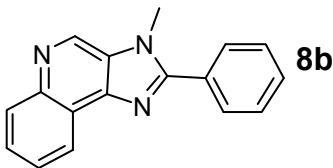
### Synthesis of Compound 7b: 2-phenyl-3-(prop-2-ynyl)-3*H*-imidazo[4,5-*c*]quinoline.



To a solution of **3b** (50 mg, 0.21 mmol) in 1 mL of anhydrous THF, were added DBU (62 mg, 0.41 mmol) and 80% propargyl bromide in toluene (98 mg, 0.82 mmol). The solution was then heated in a sealed vessel at 90 °C for an hour. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (35% EtOAc/dichloromethane) to obtain the compound **7b** (6 mg, 10%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.31 (s, 1H), 8.72 – 8.67 (m, 1H), 8.30 – 8.25 (m, 1H), 7.97 – 7.90 (m, 2H), 7.69 – 7.77 (m, 2H), 7.67 – 7.60 (m, 3H), 5.15 (d, *J* = 2.5,

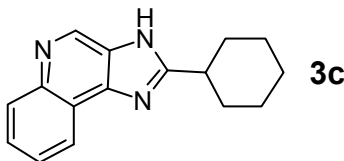
2H), 2.59 (t,  $J = 2.5$  Hz, 1H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.90, 136.03, 130.66, 129.72, 129.69, 129.17, 128.63, 127.60, 126.95, 121.90, 75.11, 35.35. MS (ESI) calculated for  $\text{C}_{19}\text{H}_{13}\text{N}_3$ ,  $m/z$  283.1109, found 284.1046 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 8b: 3-methyl-2-phenyl-3*H*-imidazo[4,5-*c*]quinoline.**



To a solution of **3b** (40 mg, 0.16 mmol) in 1 mL of anhydrous THF, were added DBU (73 mg, 0.48 mmol) and iodomethane (114 mg, 0.8 mmol). The solution was stirred for 30 minutes and the solvent was then removed under vacuum to obtain the residue, which was dissolved in EtOAc, washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (20% EtOAc/dichloromethane) to obtain the compound **8b** (15 mg, 36%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.92 – 8.85 (m, 1H), 8.75 (s, 1H), 8.53 (dd,  $J = 8.3, 1.4$  Hz, 2H), 7.83 – 7.67 (m, 3H), 7.55 – 7.44 (m, 3H), 4.29 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  170.40, 154.97, 139.30, 135.84, 134.27, 134.02, 129.75, 129.35, 128.61, 128.17, 126.36, 124.91, 121.36, 116.61, 43.88. MS (ESI) calculated for  $\text{C}_{17}\text{H}_{13}\text{N}_3$ ,  $m/z$  259.1109, found 260.1188 ( $\text{M} + \text{H}$ ) $^+$ .

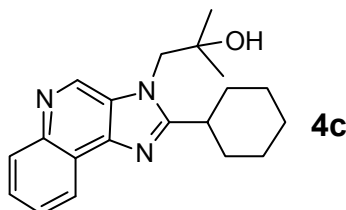
**Synthesis of Compound 3c: 2-cyclohexyl-3*H*-imidazo[4,5-*c*]quinoline.**



To a mixture of **1** (450 mg, 2.83 mmol) and cyclohexanecarboxylic acid (725 mg, 5.66 mmol), was added polyphosphoric acid (appx. 25 mL) and heated to 180 °C for an hour. The reaction mixture was then slowly cooled to room temperature and the polyphosphoric acid was slowly neutralized with ammonium hydroxide until the pH was around 8. The compound was then extracted using EtOAc and the EtOAc fraction was then washed with water, dried over sodium sulfate, concentrated and

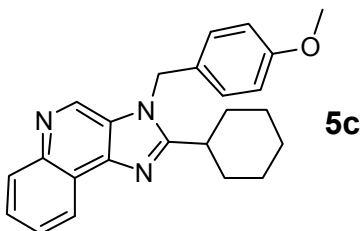
purified using column chromatography (3% MeOH/ dichloromethane) to afford the compound **3c** (409 mg, 58%).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.93 (s, 1H), 8.25 (s, 1H), 7.98 (t,  $J$  = 8.2 Hz, 1H), 7.63 – 7.52 (m, 2H), 3.02 – 2.87 (m, 1H), 2.09 – 2.01 (m, 2H), 1.83 (dd,  $J$  = 10.4, 7.3 Hz, 2H), 1.69 (dd,  $J$  = 14.8, 8.0 Hz, 1H), 1.67 – 1.59 (m, 2H), 1.46 – 1.35 (m, 2H), 1.34 – 1.22 (m, 1H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  144.54, 129.56, 128.68, 127.95, 122.57, 40.03, 32.89, 27.16, 26.91. MS (ESI) calculated for  $\text{C}_{16}\text{H}_{17}\text{N}_3$ ,  $m/z$  251.1422, found 252.1565 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 4c: 2-methyl-1-(2-cyclohexyl-3H-imidazo[4,5-c]quinolin-3-yl)propan-2-ol.**



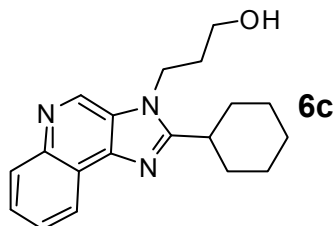
To a solution of **3c** (50 mg, 0.2 mmol) in 1 mL of 2,2-dimethyloxirane, was added DBU (61 mg, 0.4 mmol) and the solution was heated under microwave conditions (600 W, 80 °C, 1h). After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (5% MeOH/dichloromethane) to obtain the compound **4c** (42 mg, 65 %).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  9.19 (s, 1H), 8.70 – 8.56 (m, 1H), 8.14 – 8.05 (m, 1H), 7.74 – 7.61 (m, 2H), 4.43 (s, 2H), 3.33 (dt,  $J$  = 3.3, 1.6 Hz, 1H), 2.07 – 1.80 (m, 7H), 1.62 – 1.42 (m, 3H), 1.32 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  162.55, 143.37, 143.26, 137.65, 128.97, 127.93, 127.12, 126.50, 121.56, 121.47, 70.32, 53.88, 36.16, 31.55, 26.26, 25.84, 25.53. MS (ESI) calculated for  $\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}$ ,  $m/z$  323.1998, found 324.2084 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 5c: 2-cyclohexyl-3-(4-methoxybenzyl)-3H-imidazo[4,5-c]quinoline.**



To a solution of **3c** (50 mg, 0.2 mmol) in 1 mL of anhydrous THF, were added DBU (62 mg, 0.4 mmol) and 1-(chloromethyl)-4-methoxybenzene (125 mg, 0.8 mmol). The solution was then heated in a sealed vessel at 80 °C for 1 hour. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (15% EtOAc/dichloromethane) to obtain the compound **5c** (9 mg, 12%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.88 (s, 1H), 8.70 – 8.60 (m, 1H), 8.18 (dd, *J* = 6.6, 2.9 Hz, 1H), 7.72 – 7.60 (m, 2H), 7.04 (d, *J* = 8.8 Hz, 2H), 6.92 – 6.80 (m, 2H), 5.50 (s, 2H), 3.79 (s, 3H), 2.96 (s, 1H), 2.00 – 1.67 (m, 7H), 1.41 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 159.52, 135.86, 129.47, 127.60, 127.07, 126.42, 122.31, 121.95, 114.57, 55.33, 47.06, 36.74, 31.84, 26.25, 25.61. MS (ESI) calculated for C<sub>24</sub>H<sub>25</sub>N<sub>3</sub>O, *m/z* 371.1998, found 372.1990 (*M* + *H*)<sup>+</sup>.

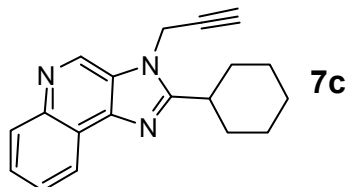
#### Synthesis of Compound **6c**: 3-(2-cyclohexyl-3*H*-imidazo[4,5-*c*]quinolin-3-yl)propan-1-ol.



To a solution of **3c** (60 mg, 0.24 mmol) in 1 mL of anhydrous DMF, were added DBU (62 mg, 0.41 mmol) and 3-bromopropan-1-ol (167 mg, 1.2 mmol). The solution was then heated in a sealed vessel at 80 °C for an hour. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (22% acetone/dichloromethane) to obtain the compound **6c** (12 mg, 16%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.18 (s, 1H), 8.66 – 8.59 (m, 1H), 8.12 (dt, *J* = 4.8, 2.8 Hz, 1H), 7.72 – 7.67 (m, 2H), 4.61 (t, *J* = 7.2 Hz, 2H), 3.64 (dd, *J* = 12.7, 7.0 Hz, 2H), 3.27 – 3.16 (m, 1H), 2.15 – 2.09 (m, 2H), 2.05 – 1.85 (m, 8H), 1.66 – 1.39 (m, 2H). <sup>13</sup>C NMR (101 MHz, MeOD) δ

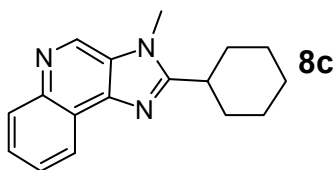
161.46, 143.61, 135.86, 128.04, 127.90, 127.22, 126.61, 121.63, 121.44, 57.72, 40.52, 35.95, 32.95, 31.62, 25.82, 25.49. MS (ESI) calculated for  $C_{19}H_{23}N_3O$ ,  $m/z$  309.1841, found 310.1927 ( $M + H$ )<sup>+</sup>.

**Synthesis of Compound 7c: 2-cyclohexyl-3-(prop-2-ynyl)-3H-imidazo[4,5-c]quinoline.**



To a solution of **3c** (60 mg, 0.24 mmol) in 1 mL of anhydrous THF, were added DBU (73 mg, 0.48 mmol) and 80% propargyl bromide in toluene (143 mg, 1.2 mmol). The solution was then heated in a sealed vessel at 90 °C for 30 minutes. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (35% EtOAc/dichloromethane) to obtain the compound **7c** (8 mg, 12%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.16 (s, 1H), 8.65 – 8.56 (m, 1H), 8.28 – 8.11 (m, 1H), 7.75 – 7.60 (m, 2H), 5.09 (d,  $J$  = 2.5 Hz, 2H), 3.00 (m, 1H), 2.48 (t,  $J$  = 2.5 Hz, 1H), 2.11 (m, 2H), 2.04 – 1.89 (m, 6H), 1.47 (m, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 159.48, 144.62, 135.35, 129.54, 127.76, 127.20, 126.51, 121.95, 76.47, 74.50, 36.58, 33.42, 31.62, 26.20, 25.65. MS (ESI) calculated for  $C_{19}H_{19}N_3$ ,  $m/z$  289.1579, found 290.1740 ( $M + H$ )<sup>+</sup>.

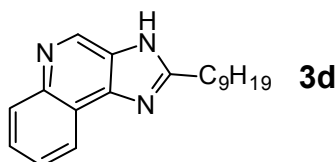
**Synthesis of Compound 8c: 2-cyclohexyl-3-methyl-3H-imidazo[4,5-c]quinoline.**



To the solution of **3c** (65 mg, 0.26 mmol) in 1 mL of anhydrous THF, were added DBU (79 mg, 0.52 mmol) and iodomethane (111 mg, 0.78 mmol) and the solution was stirred for 2 hours. The solvent was then removed under vacuum and the residue was dissolved in EtOAc, washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (20%

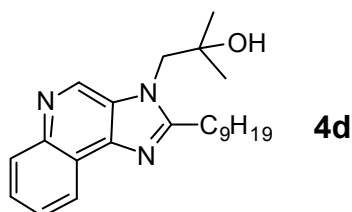
EtOAc/dichloromethane) to obtain the compound **8c** (8 mg, 12%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.03 (s, 1H), 8.70 – 8.57 (m, 1H), 8.25 – 8.13 (m, 1H), 7.74 – 7.57 (m, 2H), 3.97 (s, 3H), 3.00 – 2.94 (m, 1H), 2.17 – 1.87 (m, 7H), 1.57 – 1.37 (m, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  160.11, 144.46, 143.99, 135.12, 129.50, 128.88, 126.96, 126.37, 122.25, 121.97, 36.55, 31.38, 30.24, 26.24, 25.68. MS (ESI) calculated for  $\text{C}_{17}\text{H}_{19}\text{N}_3$ ,  $m/z$  265.1579, found 266.1666 ( $\text{M} + \text{H}$ ) $^+$ .

#### Synthesis of Compound 3d: 2-nonyl-3*H*-imidazo[4,5-*c*]quinoline.



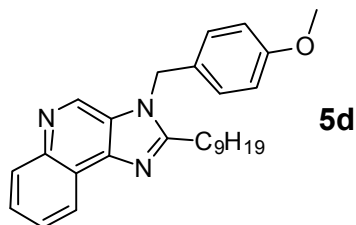
To the mixture of **1** (500 mg, 3.14 mmol) and decanoic acid (1.6 g, 9.43 mmol) was added polyphosphoric acid (appx. 5 mL) and heated under microwave conditions (1200 W, 180 °C, 30 minutes). The reaction mixture was then slowly cooled to room temperature and the polyphosphoric acid was slowly neutralized with ammonium hydroxide till the pH is around 8. The compound was then extracted using EtOAc and the EtOAc fraction was then washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (3% MeOH/ dichloromethane) to afford the compound **3d** (355 mg, 38%).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.93 (s, 1H), 8.22 (s, 1H), 8.00 (d,  $J$  = 8.5 Hz, 1H), 7.62 – 7.53 (m, 2H), 2.91 (t,  $J$  = 7.7 Hz, 2H), 1.83 – 1.75 (m, 2H), 1.36 – 1.22 (m, 4H), 1.14-1.17 (m, 8H), 0.75 (t,  $J$  = 6.9 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  158.61, 144.52, 129.56, 128.72, 128.00, 122.49, 33.02, 30.55, 30.41, 30.37, 30.32, 29.92, 29.41, 23.74, 14.45. MS (ESI) calculated for  $\text{C}_{19}\text{H}_{25}\text{N}_3$ ,  $m/z$  295.2048, found 296.2178 ( $\text{M} + \text{H}$ ) $^+$ .

#### Synthesis of Compound 4d: 2-methyl-1-(2-nonyl-3*H*-imidazo[4,5-*c*]quinolin-3-yl)propan-2-ol.



To a solution of **3d** (40 mg, 0.14 mmol) in 1 mL of 2,2-dimethyloxirane, was added DBU (62 mg, 0.41 mmol) and the solution was heated under microwave conditions (600 W, 90 °C, 1 h). After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (50% EtOAc/dichloromethane) to obtain the compound **4d** (11 mg, 18%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.17 (s, 1H), 8.58 (d, *J* = 6.4 Hz, 1H), 8.14 – 8.09 (m, 1H), 7.69 (dd, *J* = 6.8, 3.2 Hz, 2H), 4.40 (s, 2H), 3.13 (t, *J* = 6.4 Hz, 2H), 2.02 – 1.88 (m, 2H), 1.49 (d, *J* = 6.3 Hz, 2H), 1.39 (d, *J* = 15.1 Hz, 2H), 1.38 – 1.20 (m, 14H), 0.92 – 0.86 (m, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 158.89, 143.39, 143.19, 137.44, 129.30, 127.99, 127.27, 126.65, 121.52, 121.46, 70.72, 54.40, 31.66, 29.27, 29.22, 29.15, 29.05, 27.97, 27.32, 26.51, 22.37, 13.24. MS (ESI) calculated for C<sub>23</sub>H<sub>33</sub>N<sub>3</sub>O, *m/z* 367.2624, found 368.2733 (*M* + H)<sup>+</sup>.

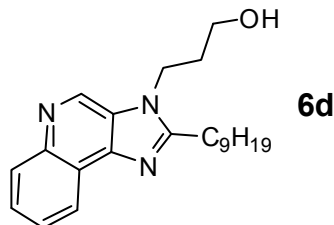
#### Synthesis of Compound 5d: 3-(4-methoxybenzyl)-2-nonyl-3*H*-imidazo[4,5-*c*]quinoline.



To a solution of **3d** (50 mg, 0.17 mmol) in 1 mL of anhydrous THF, were added DBU (52 mg, 0.34 mmol) and 1-(chloromethyl)-4-methoxybenzene (106 mg, 0.68 mmol). The solution was then heated in a sealed vessel at 80 °C for an hour. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (20% EtOAc/dichloromethane) to obtain the compound **5d** (21 mg, 30%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.89 (s, 1H), 8.66 – 8.58 (m, 1H), 8.22 – 8.15 (m, 1H), 7.73 – 7.62 (m, 2H), 7.05 (d, *J* = 8.7 Hz, 2H), 6.91 – 6.82 (m, 2H), 5.47 (s, 2H), 3.79 (s, 3H), 3.05 – 2.95 (m, 2H), 1.87 (dt, *J* = 15.6, 7.7 Hz, 2H), 1.49 – 1.38 (m, 2H), 1.33 – 1.26 (m, 10H), 0.89 (t, *J* = 6.9 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 159.57, 156.76, 144.46, 144.11, 135.74, 129.54, 128.59, 127.69, 127.33, 127.14, 126.59, 122.20, 121.75, 114.57, 55.32,

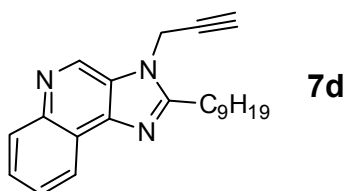
47.36, 31.85, 29.54, 29.42, 29.29, 29.25, 28.28, 27.86, 22.66, 14.11. MS (ESI) calculated for  $C_{27}H_{33}N_3O$ ,  $m/z$  415.2624, found 416.2560 ( $M + H$ )<sup>+</sup>.

**Synthesis of Compound 6d: 3-(2-nonyl-3H-imidazo[4,5-c]quinolin-3-yl)propan-1-ol.**



To a solution of **3d** (42 mg, 0.14 mmol) in 1 mL of anhydrous DMF, were added DBU (65 mg, 0.43 mmol) and 3-bromopropan-1-ol (99 mg, 0.71 mmol). The solution was then heated in a sealed vessel at 80 °C for an hour. After cooling to room temperature and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (25% acetone/dichloromethane) to obtain the compound **6d** (11 mg, 22%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.12 (s, 1H), 8.58 (dt,  $J$  = 17.0, 8.1 Hz, 1H), 8.17 (dt,  $J$  = 18.1, 9.6 Hz, 1H), 7.78 – 7.55 (m, 2H), 4.53 (t,  $J$  = 6.8 Hz, 2H), 3.72 (t,  $J$  = 5.6 Hz, 2H), 3.11 – 2.96 (m, 2H), 2.22 – 2.03 (m, 2H), 1.94 (dt,  $J$  = 15.6, 7.7 Hz, 2H), 1.54 – 1.43 (m, 2H), 1.35 – 1.28 (m, 10H), 0.89 (t,  $J$  = 6.8 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 156.89, 144.17, 144.08, 135.51, 129.19, 128.53, 127.16, 126.59, 122.15, 121.79, 58.16, 40.70, 32.85, 31.86, 29.62, 29.46, 29.36, 29.26, 28.43, 27.50, 22.66, 14.10. MS (ESI) calculated for  $C_{22}H_{31}N_3O$ ,  $m/z$  353.2467, found 354.2401 ( $M + H$ )<sup>+</sup>.

**Synthesis of Compound 7d: 2-nonyl-3-(prop-2-ynyl)-3H-imidazo[4,5-c]quinoline.**

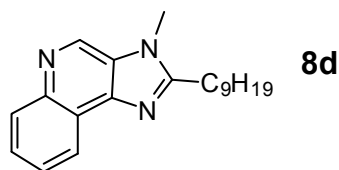


To a solution of **3d** (60 mg, 0.2 mmol) in 1 mL of anhydrous THF, were added DBU (61 mg, 0.4 mmol) and 80% propargyl bromide in toluene (119 mg, 1.0 mmol). The solution was then heated in a



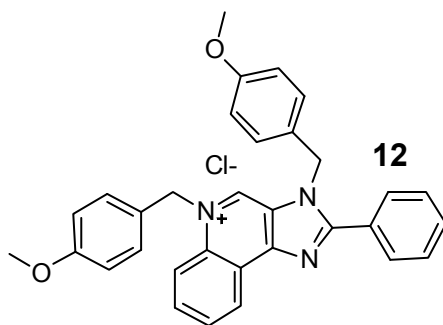
sealed vessel at 90 °C for 30 minutes. After cooling to room temperature, and removing the solvent under vacuum, the residue was dissolved in EtOAc and washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (40% EtOAc/dichloromethane) to obtain the compound **7d** (10 mg, 15%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.16 (s, 1H), 8.65 – 8.53 (m, 1H), 8.29 – 8.16 (m, 1H), 7.76 – 7.61 (m, 2H), 5.07 (d, *J* = 2.5 Hz, 2H), 3.13 – 2.97 (m, 2H), 2.48 (t, *J* = 2.5 Hz, 1H), 1.96 (dt, *J* = 15.5, 7.7 Hz, 3H), 1.57 – 1.46 (m, 2H), 1.46 – 1.38 (m, 2H), 1.30 (dd, *J* = 5.8, 4.2 Hz, 7H), 0.90 (t, *J* = 6.9 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 156.03, 144.63, 144.11, 135.27, 129.59, 127.89, 127.30, 126.70, 122.10, 121.76, 76.17, 74.63, 33.73, 31.86, 29.51, 29.44, 29.31, 29.25, 28.17, 27.67, 22.66, 14.11. MS (ESI) calculated for C<sub>22</sub>H<sub>27</sub>N<sub>3</sub>, *m/z* 333.2205, found 334.2291 (*M* + *H*)<sup>+</sup>.

#### Synthesis of Compound 8d: 3-methyl-2-nonyl-3*H*-imidazo[4,5-*c*]quinoline.



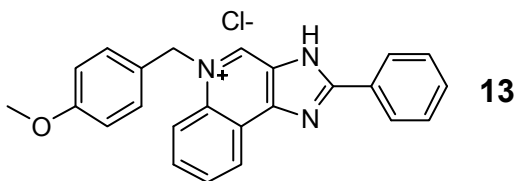
To a solution of **3d** (50 mg, 0.17 mmol) in 1 mL of anhydrous THF, were added DBU (52 mg, 0.34 mmol) and iodomethane (36 mg, 0.25 mmol) and the solution was stirred for 2 hours. The solvent was then removed under vacuum and the residue was dissolved in EtOAc, washed with water, dried over sodium sulfate, concentrated and purified using column chromatography (35% EtOAc/dichloromethane) to obtain the compound **8d** (21 mg, 40%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 9.03 (s, 1H), 8.60 – 8.56 (m, 1H), 8.19 (dt, *J* = 8.0, 4.3, 1H), 7.73 – 7.61 (m, 2H), 3.96 (s, 3H), 3.08 – 2.93 (m, 2H), 1.90 (dt, *J* = 15.6, 7.8, 2H), 1.48 (dt, *J* = 15.0, 7.0, 2H), 1.44 – 1.35 (m, 2H), 1.35 – 1.20 (m, 8H), 0.88 (t, *J* = 7.0, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 156.38, 143.89, 143.52, 134.55, 128.98, 128.49, 126.67, 126.14, 121.63, 121.31, 31.39, 30.06, 29.08, 28.99, 28.87, 28.80, 27.74, 27.22, 22.20, 13.65. MS (ESI) calculated for C<sub>20</sub>H<sub>27</sub>N<sub>3</sub>, *m/z* 309.2205, found 310.2306 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 12: 3,5-bis(4-methoxybenzyl)-2-phenyl-3*H*-imidazo[4,5-*c*]quinolin-5-ium chloride.**



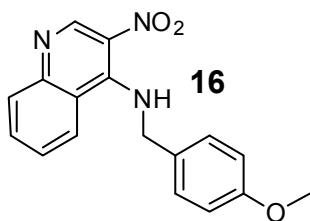
To a solution of **3b** (50 mg, 0.2 mmol) in 1 mL of anhydrous THF, were added DBU (91 mg, 0.61 mmol) and 1-(chloromethyl)-4-methoxybenzene (159 mg, 1.02 mmol), and the solution was heated under microwave conditions (600 W, 150 °C, 0.5 h). After cooling to room temperature, and removing the solvent under vacuum, the residue was purified using column chromatography (10% MeOH/dichloromethane) to obtain the compound **5b** (21 mg, 22%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.63 (s, 1H), 8.96 (d, *J* = 7.8 Hz, 1H), 8.55 (d, *J* = 9.1 Hz, 1H), 8.13 – 8.08 (m, 1H), 8.07 – 8.02 (m, 1H), 8.02 – 7.99 (m, 2H), 7.77 – 7.68 (m, 3H), 7.24 (d, *J* = 8.8 Hz, 2H), 7.10 (d, *J* = 8.7 Hz, 2H), 6.94 (d, *J* = 8.7 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.20 (s, 2H), 5.86 (s, 2H), 3.82 (s, 3H), 3.79 (s, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 163.08, 160.44, 159.97, 150.30, 135.98, 135.52, 132.27, 131.96, 129.73, 129.40, 129.10, 129.03, 128.45, 127.98, 127.57, 126.49, 124.96, 123.80, 122.18, 119.33, 114.43, 114.39, 59.82, 54.46, 54.43, 49.46. MS (ESI) calculated for C<sub>32</sub>H<sub>28</sub>N<sub>3</sub>O<sub>2</sub><sup>+</sup>, *m/z* 486.2176, found 486.2140 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 13: 5-(4-methoxybenzyl)-2-phenyl-3*H*-imidazo[4,5-*c*]quinolin-5-ium chloride.**



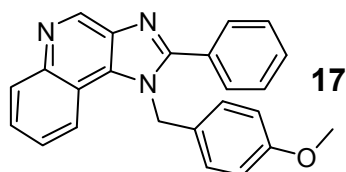
To a solution of **3b** (20 mg, 0.08 mmol) in 1 mL of anhydrous THF, was added 1-(chloromethyl)-4-methoxybenzene (19 mg, 0.12 mmol) and the solution was heated in a sealed vessel at 120 °C for an 12-14 hours. After cooling to room temperature and removing the solvent under vacuum, the residue was subjected to column chromatography (8% MeOH/dichloromethane) to obtain the compound **13** (12 mg, 40%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 9.28 (s, 1H), 8.92 – 8.84 (m, 1H), 8.41 (d, *J* = 7.0 Hz, 2H), 8.24 – 8.14 (m, 1H), 7.84 – 7.74 (m, 2H), 7.54 (dq, *J* = 14.2, 7.0 Hz, 3H), 7.23 (d, *J* = 8.6 Hz, 2H), 6.93 (d, *J* = 8.7 Hz, 2H), 6.05 (s, 2H), 3.76 (s, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 159.99, 138.62, 137.14, 134.14, 133.69, 129.64, 129.32, 128.37, 128.00, 127.75, 126.66, 123.91, 121.38, 118.59, 114.25, 58.48, 54.35. MS (ESI) calculated for C<sub>24</sub>H<sub>20</sub>N<sub>3</sub>O<sup>+</sup>, *m/z* 366.1601, found 366.1760 (*M* + H)<sup>+</sup>.

#### Synthesis of Compound 16: *N*-(4-methoxybenzyl)-3-nitroquinolin-4-amine.



To a solution of **14** (147 mg, 0.71 mmol) in 4:1 mixture of toluene and 2-propanol were added *N,N*-diethylpropan-2-amine (0.14 mL, 6 mmol) and **15** (121 mg, 0.88 mmol). The reaction mixture was heated to 70 °C for half an hour until a solid started to precipitate. The reaction mixture was then cooled, filtered and washed with toluene/2-propanol (7:3), ether and cold water. The residue was dried at 80 °C to obtain the compound **16** (200 mg, 91%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.82 (s, 1H), 9.41 (s, 1H), 8.34 (d, *J* = 8.4, 1H), 8.03 (d, *J* = 8.3, 1H), 7.79 (dd, *J* = 11.2, 4.1 Hz, 1H), 7.48 (dd, *J* = 11.3 Hz, 4.2, 1H), 7.34 (d, *J* = 8.6 Hz, 2H), 6.97 (d, *J* = 8.6 Hz, 2H), 5.07 (d, *J* = 5.4 Hz, 2H), 3.85 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 159.76, 150.82, 150.64, 147.43, 132.72, 130.46, 128.83, 128.68, 126.99, 126.20, 125.52, 119.22, 114.72, 55.37, 52.74. MS (ESI) calculated for C<sub>17</sub>H<sub>15</sub>N<sub>3</sub>O<sub>3</sub>, *m/z* 309.1113, found 310.1268 (*M* + H)<sup>+</sup>.

**Synthesis of Compound 17: 1-(4-methoxybenzyl)-2-phenyl-1*H*-imidazo[4,5-*c*]quinoline.**



Compound **16** (120 mg, 0.39 mmol) was dissolved in MeOH and hydrogenated over Pd/C as a catalyst at 60 psi hydrogen pressure for 4 hours. The solution was then filtered using celite, followed by evaporation of the solvent under reduced pressure to afford *N*<sup>4</sup>-(4-methoxybenzyl)quinoline-3,4-diamine (85 mg, 94%). *N*<sup>4</sup>-(4-methoxybenzyl)quinoline-3,4-diamine (85 mg, 0.31 mmol), benzoic acid (41 mg, 0.34 mmol), HBTU (129 mg, 0.34 mmol), triethylamine (34 mg, 0.34 mmol) and a catalytic amount of DMAP were dissolved in 5 mL of DMF and stirred for 10-12 hours. The solvent was then removed under vacuum. The residue was dissolved in EtOAc and washed with water, dried over sodium sulfate and concentrated under reduced pressure to obtain the residue, which was dissolved in 10 mL of ethanol, and a solution of excess of sodium hydroxide in 1 mL of water was added. The reaction mixture was refluxed for 5-6 hours and then the solvent was removed to obtain the residue, which was purified using column chromatography (4% MeOH/dichloromethane) to obtain the compound **17** (82 mg, 80%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.47 (s, 1H), 8.31 (d, *J* = 7.7 Hz, 1H), 7.94 (d, *J* = 7.7 Hz, 1H), 7.71 (dd, *J* = 8.1 Hz, 1.4, 2H), 7.68 – 7.62 (m, 1H), 7.58 – 7.42 (m, 4H), 7.10 (d, *J* = 8.7 Hz, 2H), 6.95 – 6.91 (m, 2H), 5.81 (s, 2H), 3.82 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 159.35, 154.84, 145.54, 137.06, 130.86, 130.33, 129.46, 128.91, 127.76, 127.07, 126.71, 126.52, 120.53, 117.76, 114.91, 55.30, 50.12. MS (ESI) calculated for C<sub>24</sub>H<sub>19</sub>N<sub>3</sub>O, *m/z* 365.1528, found 366.1785 (*M* + H)<sup>+</sup>.

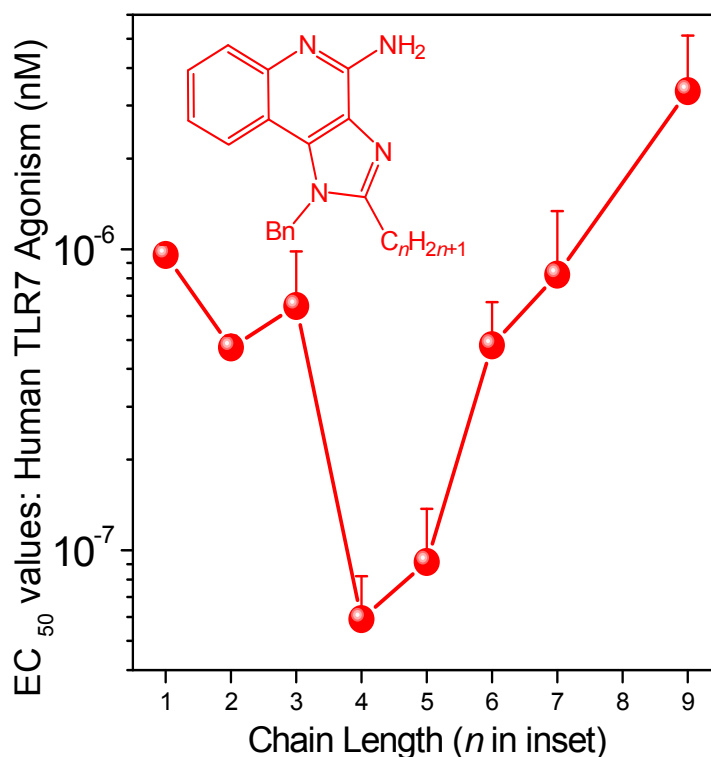
**2D-NOESY experiments:** The 2D-NOESY experiments were performed on the Bruker Avance 400 or Avance AV-III 500 NMR instruments. Compounds were dissolved in appropriate deuterated

solvents and experiments were performed with mixing time (d8) of 0.5 sec (400 MHz) or 0.7 sec (500 MHz). The data generated was processed using MestReNova 6.2.1 (Mestrelab Research S.L.).

**TLR-7/8 antagonism assay:** A reporter gene assay using TLR7<sup>132;137;138</sup> (or TLR8)-dependent NF- $\kappa$ B induction was used. The inhibition of induction of NF- $\kappa$ B, a key transcriptional activator of the innate immune system, was quantified using human embryonic kidney 293 cells stably transfected with plasmids encoding TLR7 as well as an NF- $\kappa$ B reporter gene coupled to secreted alkaline phosphatase (sAP) (InvivoGen, San Diego, CA), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF- $\kappa$ B/AP-1 promoters is inducible by the gardiquimod (TLR7 agonist) or CL075 (TLR8 agonist), and extracellular sAP in the supernatant is proportional to NF- $\kappa$ B induction. HEK-Blue-7 (or HEK-Blue-8) cells were incubated at a density of  $\sim 10^5$  cells/mL in a volume of 80  $\mu$ L/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently stimulated with 1  $\mu$ g/mL of gardiquimod or CL075. Concurrent to stimulation, serially diluted concentrations of test compounds were added to the cell medium using a rapid-throughput, automated protocol employing a Bio-Tek P2000 liquid handler and left to incubate overnight. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

## Chapter 4.

# STRUCTURE-ACTIVITY RELATIONSHIPS IN HUMAN TOLL-LIKE RECEPTOR 7-ACTIVE IMIDAZOQUINOLINE ANALOGUES



## 4.1. Introduction.

As discussed in Chapter 1, we have recently examined representative members of virtually the entire compendium of known TLR agonists in a series of hierarchical assays including primary TLR-reporter assays, secondary indices of immune activation such as cytokine induction and activation of lymphocytic subsets in whole human blood, and tertiary screens characterizing transcriptomal activation patterns with a view to identifying optimal immunostimulatory chemotypes.<sup>69</sup> Of all the innate immune stimuli examined, we found that TLR7 agonists were extraordinarily immunostimulatory, stimulating virtually all subsets of lymphocytes, and yet without inducing dominant proinflammatory cytokine responses.<sup>69</sup> We therefore became especially desirous of evaluating TLR7-active compounds as potential vaccine adjuvants.

Long before endosomal TLR7 was discovered to serve as the primary sensor for short, single-stranded, GU-rich RNA sequences (ssRNA), mainly of viral origin,<sup>70-72</sup> a number of small molecules were synthesized and evaluated in the 1970s and '80s for antiviral activities owing to their pronounced Type I interferon (IFN- $\alpha$  and - $\beta$ ) inducing properties.<sup>139-143</sup> Although the mechanisms of innate immune stimulation of several of these compounds (such as tilorone<sup>141</sup> and bromopirone<sup>143</sup>) remain yet to be formally elucidated, members of the 1*H*-imidazo[4,5-*c*]quinolines were found to be good Type I IFN inducers in human cell-derived assays,<sup>144</sup> and FDA approval was obtained in 1997 for Imiquimod (**1**, **Fig. 1**) for the treatment of basal cell carcinoma and actinic keratosis.<sup>145</sup> It was not until 2002, however, that the mechanistic basis of IFN induction by the imidazoquinolines was found to be a consequence of TLR7 engagement and activation.<sup>119</sup> Other than the original landmark studies performed by investigators at 3M Pharmaceuticals,<sup>144</sup> structure-activity relationships of the imidazoquinoline chemotype remains poorly explored, perhaps attributable in part to recent interest in the 8-hydroxy-adenine compounds as alternate TLR7-agonists,<sup>146-149</sup> which appear to lack emetic side-effects observed in ferrets upon oral administration.<sup>148</sup>

An aspect of our recent work in this area<sup>69;137;150</sup> focuses on developing adjuvants for transcutaneous vaccines (needle-free vaccine patches).<sup>151-153</sup> Given that imiquimod is already approved for topical use, it was of particular interest not only to explore chemical space around the imidazoquinolines, but also to carefully ‘immunophenotype’ such compounds in human TLR7-based assays to verify that they did not have any TLR8-driven proinflammatory properties. One of our goals was also to learn from such structure-activity studies optimal positions on the scaffold that would tolerate the introduction of electrophilic or photoactivable labels for purposes of developing self-adjuvanting vaccine constructs<sup>154;155</sup> by covalently modifying protein antigens. We report here the synthesis and evaluation of a focused library of variously substituted 1*H*-imidazo[4,5-*c*]quinolines, and the identification of a novel, pure TLR7 agonist whose potency is 250 times that of imiquimod.

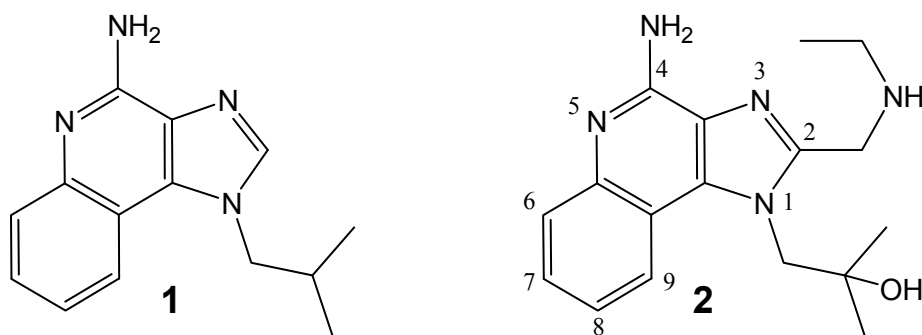
## 4.2. Results and Discussion.

As alluded to earlier, long before the fact that imidazoquinolines specifically engaged and activated TLR7 was known, it was recognized that these compounds were potently adjuvant<sup>77-79;81</sup> in addition to displaying anti-viral effects via the induction of IFN- $\alpha/\beta$ .<sup>83;84</sup> In human blood stimulated *ex vivo* with TLR7 agonists, upregulation of the surface expression of costimulatory molecules such as CD40, CD80, and CD86 occurred in both CD11c<sup>-</sup> plasmacytoid DCs and CD11c<sup>+</sup> myeloid DCs; furthermore, the Th1 stimulatory ability of both DC subsets was enhanced in response to TLR7 ligands.<sup>85</sup> Because TLR7 agonists transmit a T-helper-like signal to antibody-producing B cells, it is a highly effective adjuvant even for synthetic peptides that lack T-cell epitopes, possibly even replacing the function of T-helper cells, and providing a potential T-cell-independent vaccination strategy.<sup>81</sup>



Our initial interest in exploring TLR7 agonists as vaccine adjuvants has been greatly reinforced by our recent observations that pure TLR7 agonists, unlike other TLR ligands, are potently immunostimulatory without activating inflammatory programs in human whole blood model systems.<sup>69</sup> We elected to choose 1-(4-amino-2-((ethylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol [gardiquimod, **2** (**Fig. 1**)] as our point of departure in examining structure-activity relationships in the imidazoquinolines, rather than imiquimod because we had earlier established that this analogue was more active than the parent compound, and was also verified to be a pure TLR7 agonist in human primary cells.<sup>69;137</sup>

*Fig. 1. Structures of Imiquimod (1) and Gardiquimod (2)*



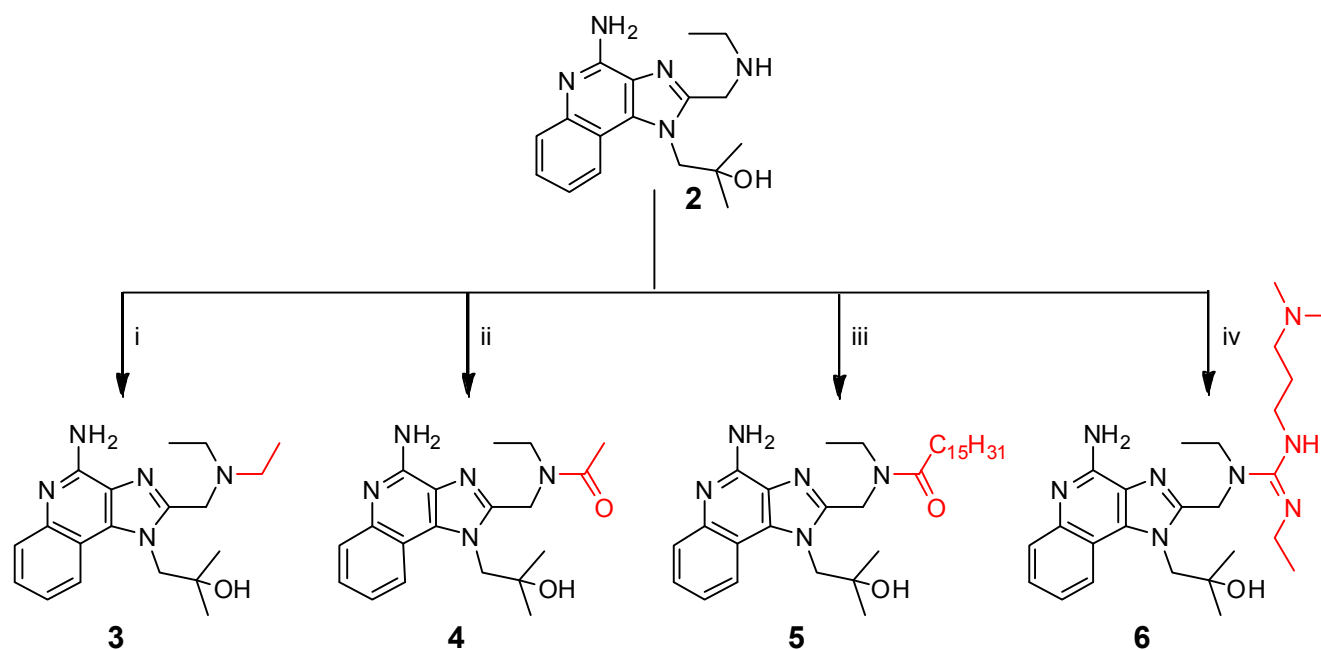
The synthesis of **2** has been described in Chapter 2.<sup>137</sup> The only report on a detailed examination of SAR on the imidazoquinolines by the 3M group<sup>144</sup> had used indirect bioassays (indirect assay for IFN- $\alpha$  using inhibition of virus-induced cytopathic effect) which predated the discovery and development of TLR7-specific assays. In the studies reported below, all compounds have been evaluated in human TLR7 and TLR8 reporter gene assays; highly active compounds were further examined for IFN- $\alpha$  induction in whole human blood using analyte-specific immunoassays.

Recent studies show that the TLR7 agonistic imidazoquinolines are indeed potently adjuvant in vaccine constructs,<sup>156;157</sup> however, the route of administration appears to be crucial since intradermal, but not subcutaneous administration of the vaccine adjuvanted with **2** is immunogenic and protective.<sup>156</sup> Epidermal Langerhans cells as well as dermal dendritic cells

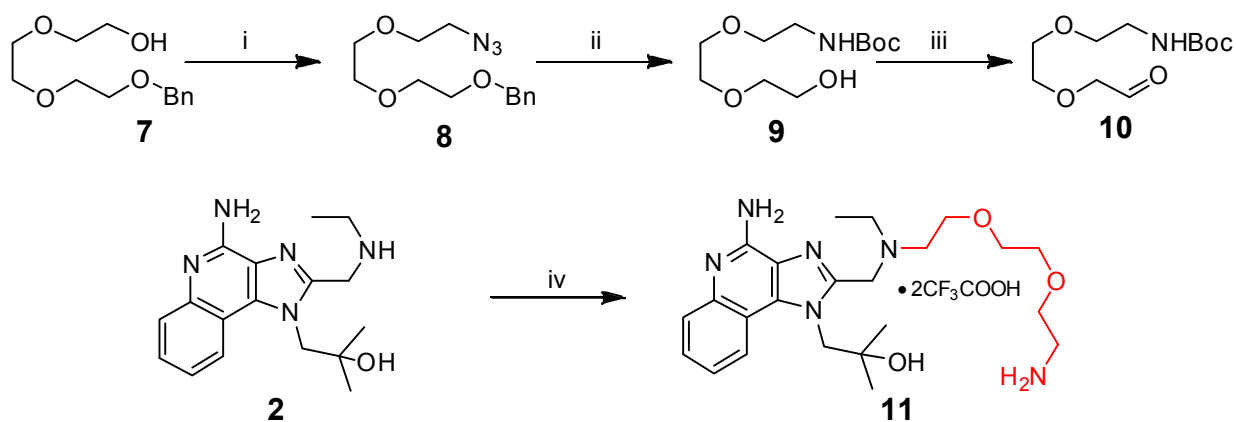
(myeloid and plasmacytoid subsets)<sup>158</sup> function as specialized professional antigen presenting cells in the skin.<sup>159;160</sup> The plasmacytoid dendritic cell subset appears to be the target cell-type for TLR7 agonists.<sup>161</sup> We hypothesized that the C2 ethylaminomethylene substituent of **2** renders the molecule sufficiently polar as to hinder transcutaneous penetration to effectively access and stimulate plasmacytoid dendritic cells. It was therefore desirable to evaluate more hydrophobic analogues of **2** as potential candidate transcutaneous vaccine adjuvants.

Unlike TLR2, TLR3, and TLR4 for which crystal structures are available as complexes with their cognate ligands,<sup>162</sup> a detailed structural characterization of the mode of binding of the imidazoquinolines to TLR7 is not yet available. Therefore, our strategy in examining structure-activity relationships was focused rather on exploring chemical space systematically around the validated lead compound **2**, toward realizing the two major objectives mentioned earlier. We began with derivatization of the secondary amine of the C2 ethylaminomethylene sidechain in an effort to examine whether this could be a potential site for introducing photoactivable or electrophilic functionalities. We found that modifications on this substituent are poorly tolerated, with only the *N*-ethyl analogue **3** (Scheme 1) retaining partial activity, and acyl (**4**, **5**) and guanidine<sup>163</sup> (**6**) derivatives being bereft of activity (Table 1).

**Scheme 1.** Syntheses of 2-imidazolyl sidechain-modified analogues of **2**.



Reagents: i.  $\text{CH}_3\text{CHO}$ ,  $\text{NaCNBH}_3$ ,  $\text{MeOH}$ ; ii.  $\text{CH}_3\text{COCl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{THF}$ ; iii.  $\text{C}_{15}\text{H}_{31}\text{COCl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{THF}$ ; iv.  $\text{EDCI}$ ,  $\text{Et}_3\text{N}$ ,  $\text{DMF}$ .

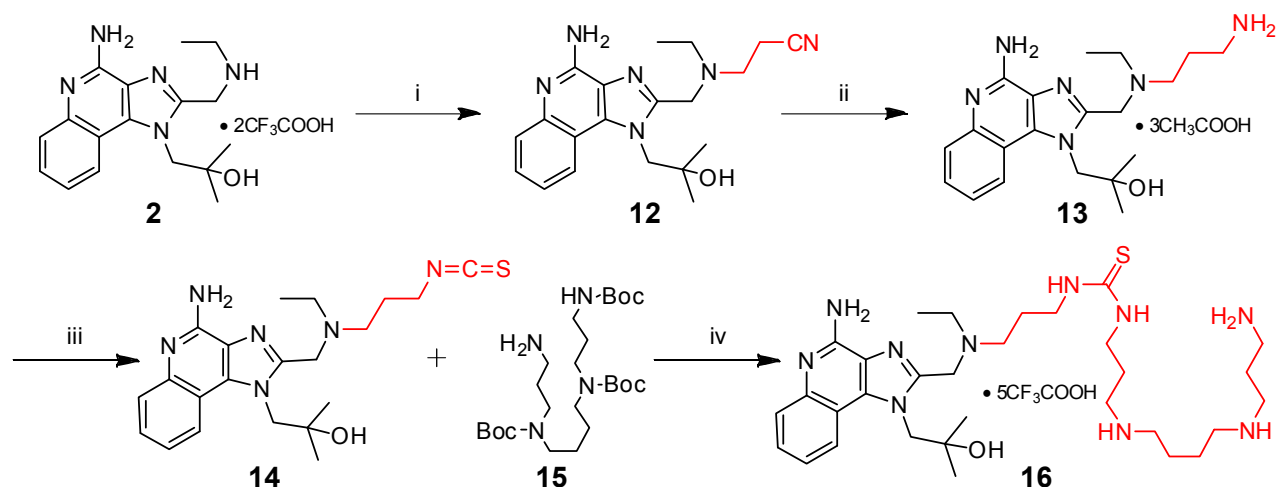


Reagents: i. (a)  $\text{TsCl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{DMAP}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $0^\circ\text{C}$  (b)  $\text{NaN}_3$ ,  $\text{DMF}$ ,  $70^\circ\text{C}$ ; ii.  $(\text{Boc})_2\text{O}$ ,  $\text{Pd}(\text{OH})_2/\text{C}$ ,  $\text{H}_2$ ,  $\text{MeOH}$ ; iii. Dess martin periodinane,  $\text{CH}_2\text{Cl}_2$ ; iv. (a) **10**,  $\text{NaCNBH}_3$ ,  $\text{MeOH}$  (b)  $\text{CF}_3\text{CO}_2\text{H}$ .

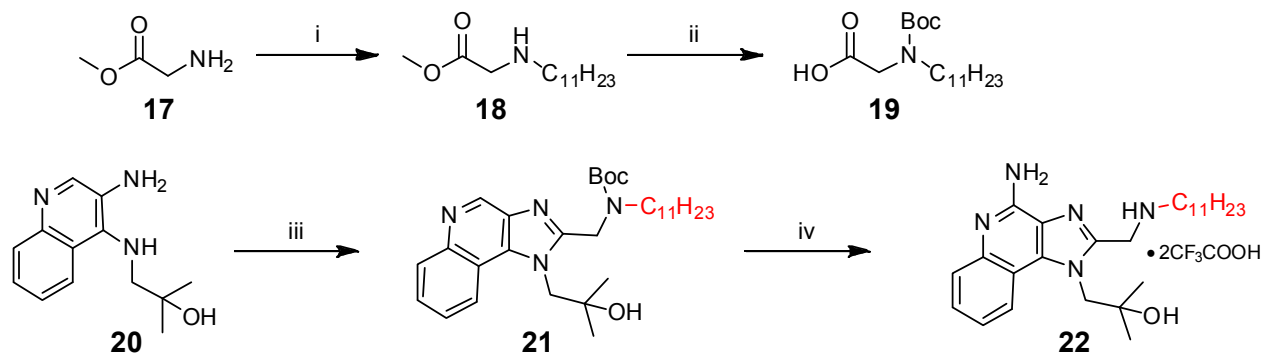
The loss of activity appeared not to be a consequence of altered basicity of the amine; surmising that it may, in part, be due either to the hydrophobicity of the substituent, or the conformational constraint imposed by the amide or guanidine, we asked if a polar spacer group, such as triethyleneglycol (**11**) may mitigate loss of activity, but we were proven wrong because **11** was also found to be completely inactive. Concurrent with the syntheses of the above compounds, we also synthesized the *N*-propyleneisothiocyanate derivative (**14**) via the *N*-propyleneamino (**13**) and *N*-ethylenenitrile (**12**) precursors. Compound **14** was not tested in reporter gene assays because we anticipated spurious results on account of its reactivity; the reaction of **14** with the spermine derivative (used as a model amine) afforded the expected thiourea adduct (**16**) which, disappointingly, was also inactive. These results conclusively showed that the secondary amine on the C2 substituent was not amenable to modifications. Replacement of the terminal ethyl group on the C2 substituent with an *n*-undecanyl group in **22** resulted in complete loss of activity (Scheme 2, Table 1), signaling that the length of the C2 substituent was crucial; this was to become dramatically apparent when **32**, with a pentyl substituent on C2 was evaluated later (see below).

Desiring more lipophilic analogues which are expected to permeate the dermal barrier better, we sought to examine *N*<sup>1</sup>-benzyl analogues (Scheme 3) noting that similar substitutions have resulted in augmented activity in the 8-hydroxy-adenine series.<sup>146-149</sup> Compound **32** was the first to be synthesized in this series which, to our pleasant surprise showed an activity twenty-fold greater than **2**. Coupled with our observation that **22** was completely inactive, we sought to systematically explore SAR of the *N*<sup>1</sup>-benzyl C2 alkyl substituents. As depicted in **Fig. 2**, a very distinct relationship between alkyl length and TLR7-agonistic potency was observed, with the optimal compound being **31** (C2-*n*-butyl), with an EC<sub>50</sub> of 59 nM (Table 1). *n*-Butylene (**38**) and *n*-butyne (**43**) analogues showed decrease in potency. It is noteworthy that a C2-*n*-butyl, *N*<sup>1</sup>-phenyl analogue synthesized by the 3M group<sup>144</sup> was not the most active; our results indicate that an *N*<sup>1</sup>-benzyl substituent is preferred.

**Scheme 2.** Syntheses of 2-imidazolyl sidechain-modified analogues of **2**.

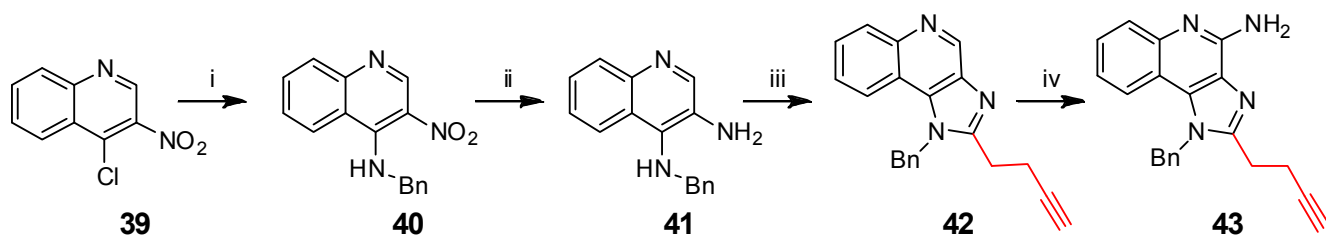
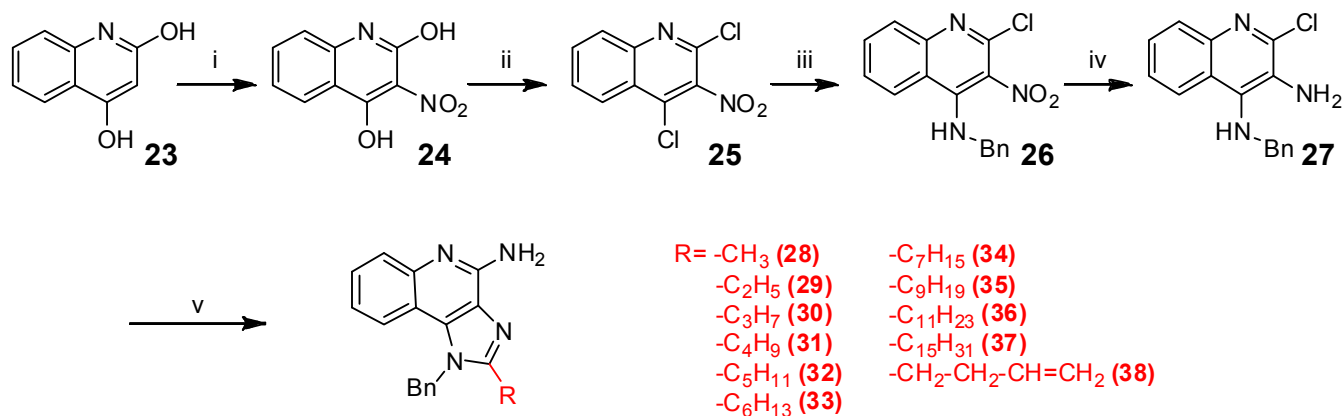


Reagents: i.  $\text{CH}_2=\text{CHCN}$ ,  $\text{Et}_3\text{N}$ ,  $\text{MeOH}$ ; ii.  $\text{Pd}(\text{OH})_2/\text{C}$ ,  $\text{H}_2$ ,  $\text{CH}_3\text{CO}_2\text{H}$ ; iii.  $\text{CS}_2$ ,  $\text{Et}_3\text{N}$ ,  $(\text{Boc})_2\text{O}$ ,  $\text{EtOH}$ ; iv.  $\text{CH}_2\text{Cl}_2$ .



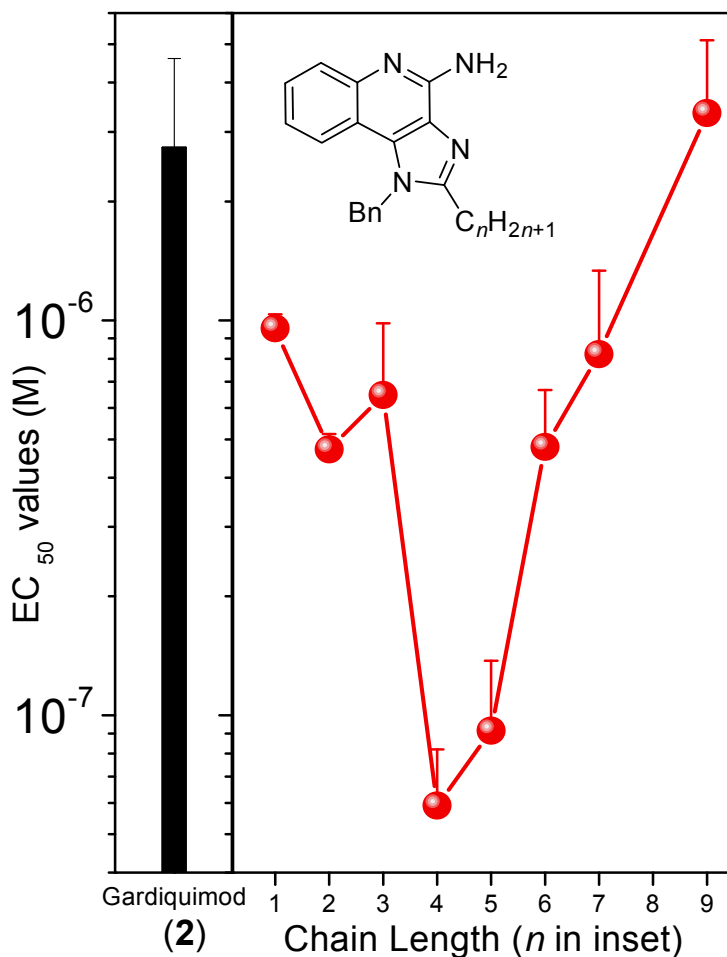
Reagents: i.  $\text{C}_{10}\text{H}_{21}\text{CHO}$ ,  $\text{NaCNBH}_3$ ,  $\text{MeOH}$ ; ii. (a)  $(\text{Boc})_2\text{O}$ ,  $\text{Et}_3\text{N}$ ,  $\text{MeOH}$  (b)  $\text{LiOH}$ ,  $\text{THF}/\text{MeOH}$ ; iii. (a) **19**, HATU,  $\text{Et}_3\text{N}$ , DMAP,  $\text{DMF}$  (b)  $\text{NaOH}$ ,  $\text{EtOH}$ , reflux; iv (a) 3-chloroperoxybenzoic acid,  $\text{CH}_2\text{Cl}_2$ ,  $\text{CHCl}_3$ ,  $\text{MeOH}$  (b) benzoyl isocyanate,  $\text{CH}_2\text{Cl}_2$  (c)  $\text{NaOCH}_3$ ,  $\text{MeOH}$  (d)  $\text{CF}_3\text{CO}_2\text{H}$ .

**Scheme 3.** Syntheses of 1-benzyl-2-(alkyl)-1H-imidazo[4,5-c]quinolin-4-amines.

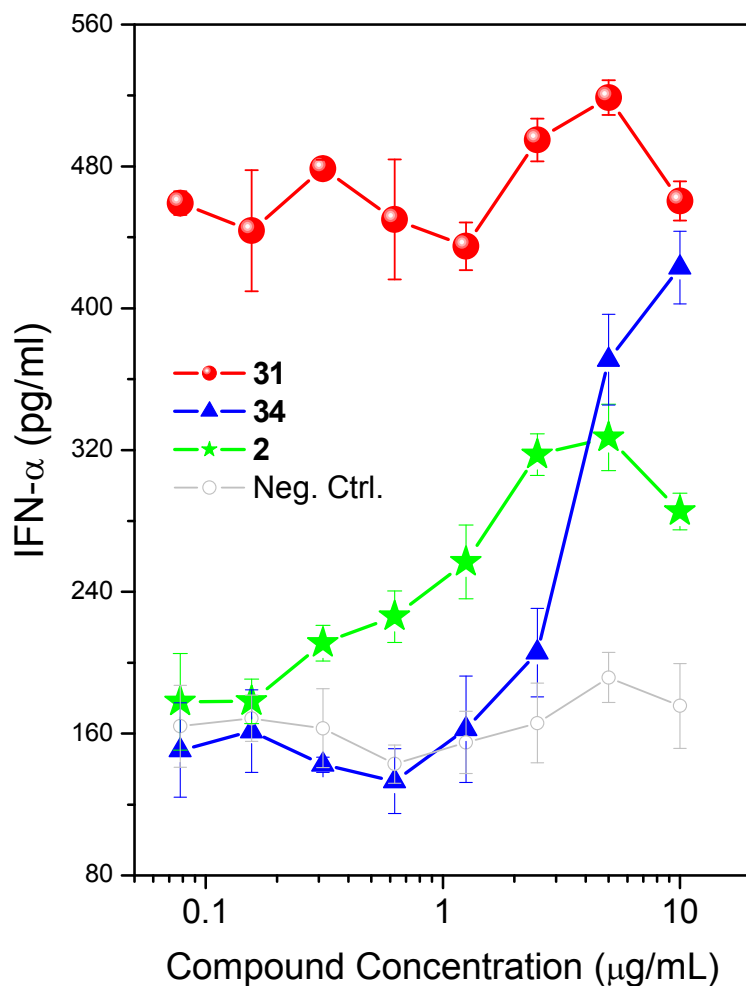


In recognition that bioactivity readouts using cell-culture systems may not always reflect with fidelity in vivo behavior owing to differential plasma protein binding (that we ourselves have observed and characterized),<sup>164</sup> it was important to verify that the activity profiles observed in TLR7-specific reporter gene assays (Table 1) was also seen in whole blood with its full complement of plasma proteins. It was gratifying that we observed the expected **31**>>**2**~**34** activity profile in whole blood IFN- $\alpha$  induction experiments (**Fig. 3**).

**Fig. 2.** Alkyl chain length dependence of TLR7-agonistic activity profiles of the N<sup>1</sup>-benzyl-C2-alkyl compounds (right panel). The activity of **2** (reference compound) is shown on the left. Error bars represent standard deviations obtained on quadruplicate samples.



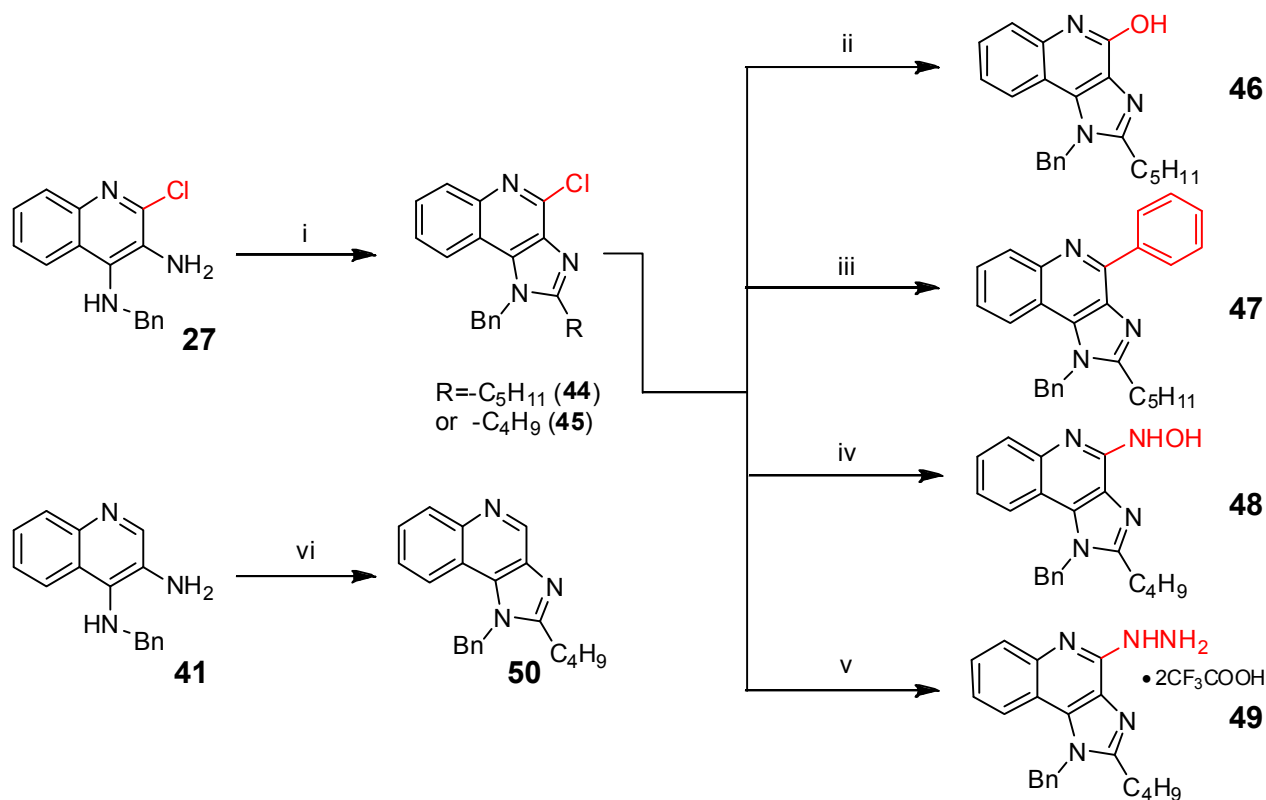
**Fig. 3.** Dose-dependent induction of IFN- $\alpha$  in whole human blood by **2**, **31**, and **34**. Plasma IFN- $\alpha$  was assayed in triplicate by ELISA.



Bioisosteric replacements of the C4-NH<sub>2</sub> group with -NMe, -OH and -SMe groups on the imiquimod scaffold have been explored earlier,<sup>144</sup> and it was of interest to examine if the previously-described SAR would hold true in our analogues, and also to test whether the introduction of phenyl, -NHNH<sub>2</sub> and -NHOH groups would alter TLR7 activity (Scheme 4). The 4-Cl precursor (**44**) and the des-amino analogue **50**, as well as the 4-OH (**46**) and 4-Ph (**47**) analogues were completely inactive, while the 4-NHOH (**48**) and 4-NHNH<sub>2</sub> (**49**) compounds were substantially weaker (Table 1) than their 4-NH<sub>2</sub> counterparts (**31**, **32**). These results indicate the importance of the preservation of the NH<sub>2</sub> group on C4 for maximal activity.



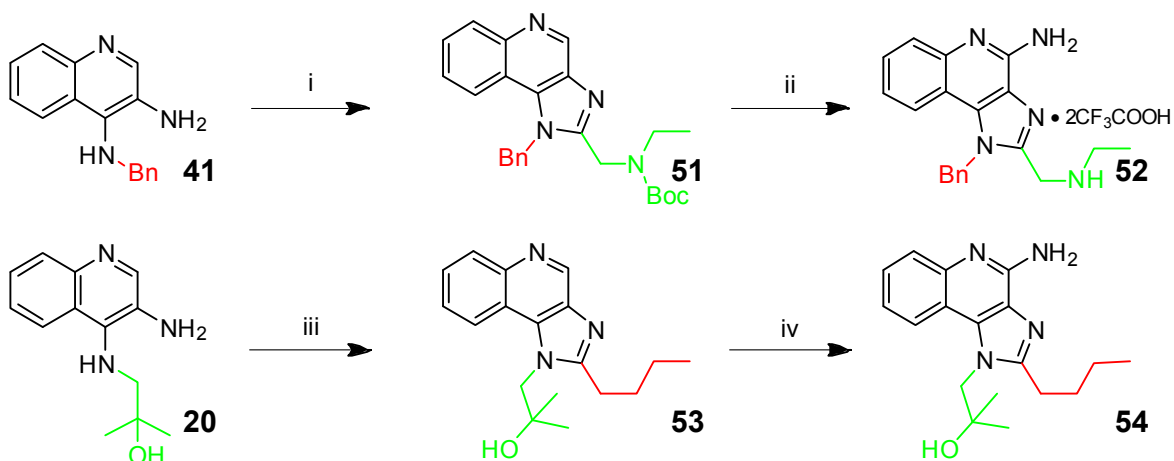
**Scheme 4.** Syntheses of 4-substituted-1-benzyl-2-(alkyl)-1H-imidazo[4,5-c]quinolines.



Reagents: i. (a) RCOCl, Et<sub>3</sub>N, THF (b) CaO, MeOH, 110° C; ii. 6N aq. HCl, 110° C; iii. polystyrene-bound PPh<sub>3</sub>-Pd, phenyl boronic acid, K<sub>2</sub>CO<sub>3</sub>, THF/H<sub>2</sub>O; iv. NH<sub>2</sub>OH.HCl, Et<sub>3</sub>N, MeOH, 50° C; v. (a) *t*-butyl carbazate, 80° C, MeOH (b) CF<sub>3</sub>CO<sub>2</sub>H, vi. (a) RCOCl, Et<sub>3</sub>N, THF (b) CaO, MeOH, 110° C.

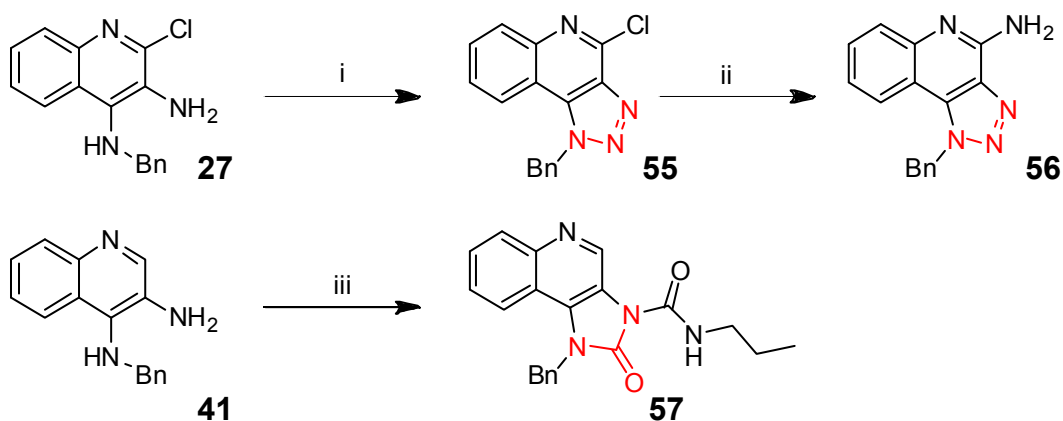
Up to this point in our SAR studies, the most active and chemically distinct species were compound **2**, and the *N*<sup>1</sup>-benzyl-C2-*n*-butyl analogue **31**. We were curious to examine whether transposing the *N*<sup>1</sup> and C2 substituents on these molecules would result in enhanced activity. Fortuitously, both the 'hybrids' (**52**, **54**; Scheme 5) were very active. **54** was found to be extraordinarily potent with an EC<sub>50</sub> of 8.6 nM (**Fig. 4**, Table 1).

**Scheme 5.** Syntheses of C-2/N-1 substituent-swapped **31/2** hybrids.



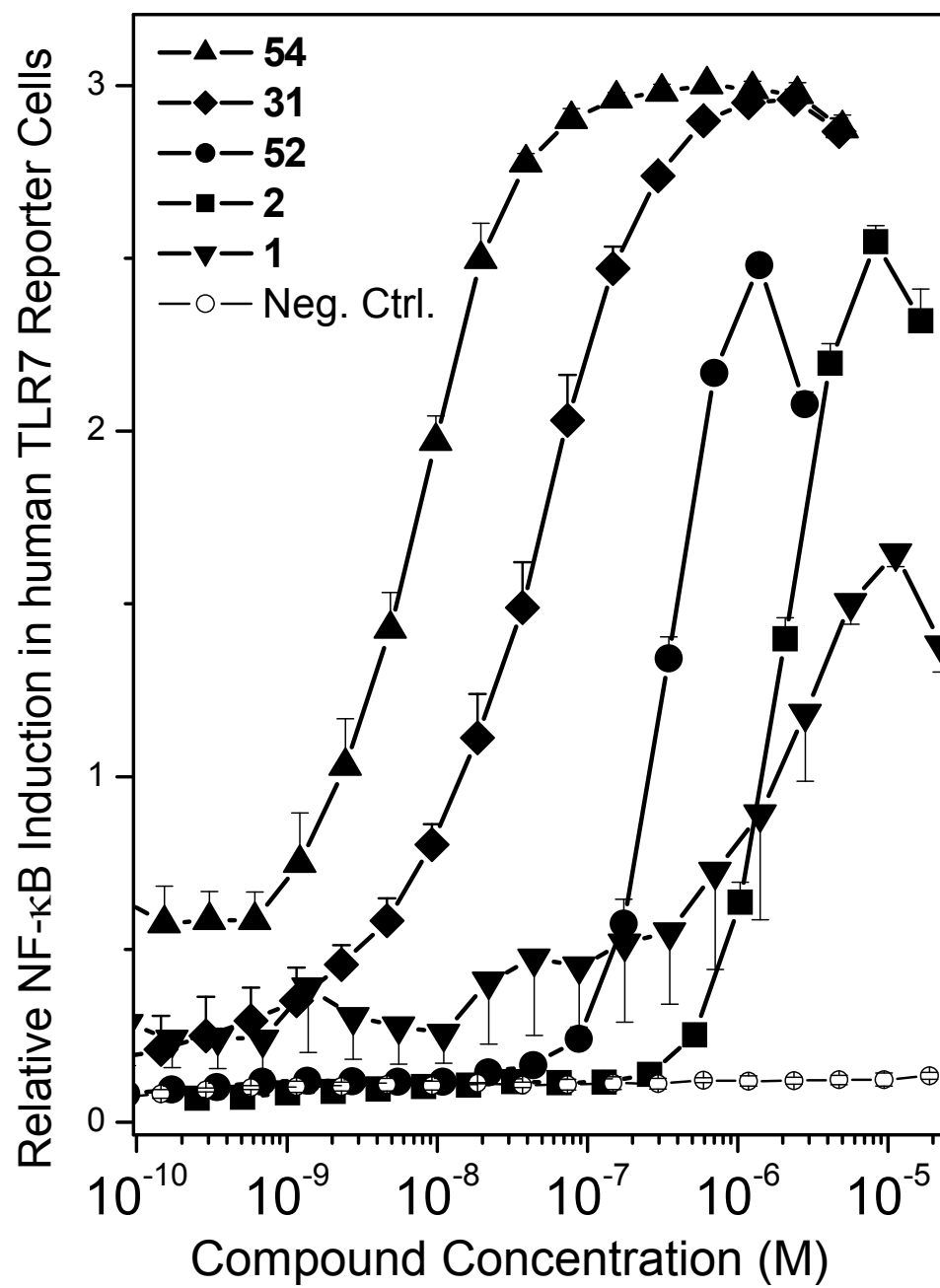
Reagents: i. HBTU, 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid, Et<sub>3</sub>N, DMAP, DMF, 70° C; ii. (a) 3-chloroperoxybenzoic acid, CH<sub>2</sub>Cl<sub>2</sub>, CHCl<sub>3</sub>, MeOH (b) benzoyl isocyanate, CH<sub>2</sub>Cl<sub>2</sub> (c) NaOCH<sub>3</sub>, MeOH (d) CF<sub>3</sub>CO<sub>2</sub>H; iii. (a) C<sub>4</sub>H<sub>9</sub>COCl, Et<sub>3</sub>N, THF (b) CaO, MeOH, 110° C; iv. (a) 3-chloroperoxybenzoic acid, CH<sub>2</sub>Cl<sub>2</sub>, CHCl<sub>3</sub>, MeOH (b) benzoyl isocyanate, CH<sub>2</sub>Cl<sub>2</sub> (c) NaOCH<sub>3</sub>, MeOH.

**Scheme 6.** Syntheses of imidazole-modified analogues.



Reagents: i. NaNO<sub>2</sub>, CH<sub>3</sub>CO<sub>2</sub>H, H<sub>2</sub>O, 0° C; ii. NH<sub>3</sub>/MeOH, 150° C; iii. *n*-Propyl isocyanate, Et<sub>3</sub>N, 100° C, THF.

**Fig. 4.** TLR7-agonistic activities of active compounds in a human TLR7-specific reporter gene assay. Error bars represent standard deviations obtained on quadruplicate samples.

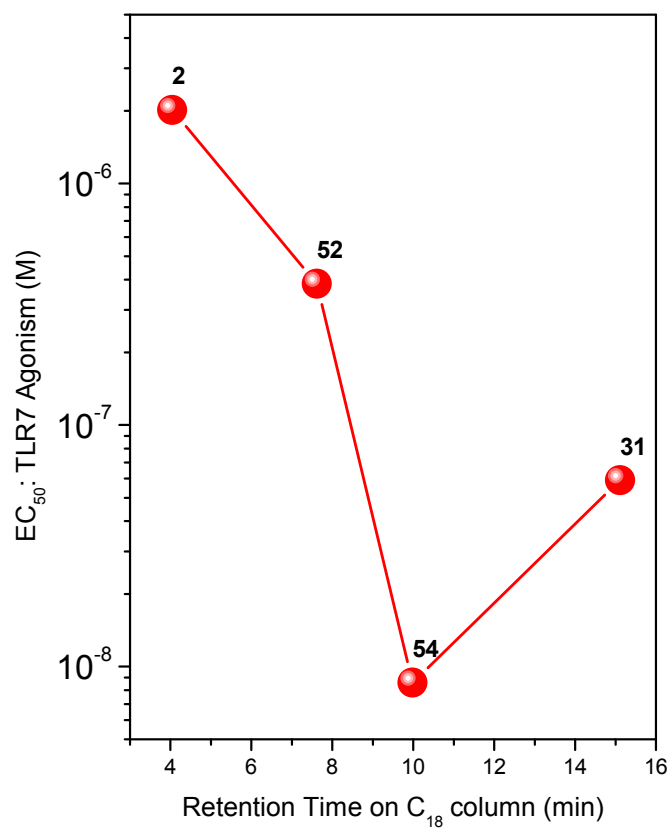


Finally, we attempted to modify the imidazole ring of the scaffold. The triazole compounds **55** and **56**, as well as the cyclic urea **57** were completely inactive, which emphasizes the role of the recognition of the imidazole ring system by TLR7. Mention must also be made that these studies have been useful in delineating possible sites for introducing labeling functionalities. Preliminary studies (data not shown) show that the *N*<sup>1</sup>-benzyl substituent may be appropriate for introducing arylazido functional groups, while the quinoline ring may be amenable to introducing electrophilic groups. These results will be communicated in a future publication.

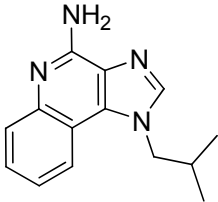
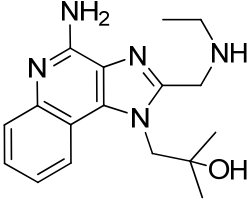
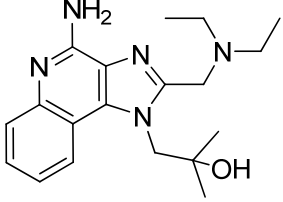
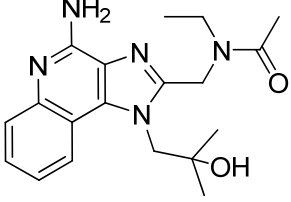
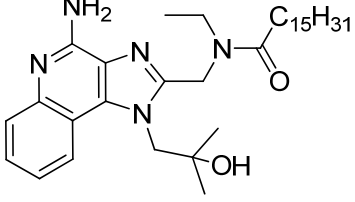
All of the C2-alkyl compounds reported herein were tested for TLR8 activity using the thiazoloquinoline CL075 (3M002) as a reference TLR8 agonist,<sup>165;166</sup> and none had any appreciable activity in these assays. It should also be pointed out that while potencies of compounds are conventionally represented as EC<sub>50</sub> (or IC<sub>50</sub>) values, that metric alone appears to be inadequate in rank-ordering the activities of the active compounds tested. An inspection of **Fig. 4** indicates, for instance, that while the EC<sub>50</sub> values of both imiquimod and **2** are comparable (~ 2 μM), compound **2** induces a more robust response than imiquimod. Similarly, the area-under-curve estimates of **54** (analogous to combined 'avidity' and affinity measures of antigen-antibody interactions)<sup>167;168</sup> indicate that consideration of the EC<sub>50</sub> values alone may underestimate its potency.

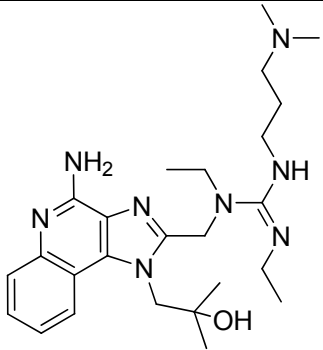
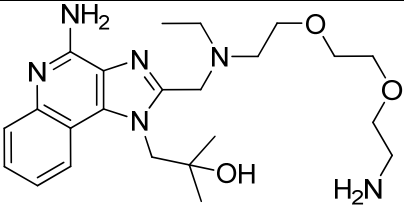
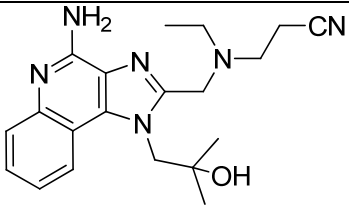
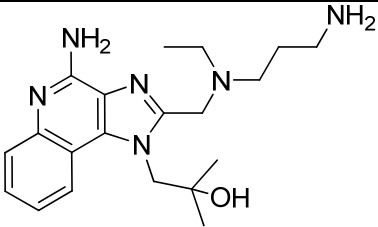
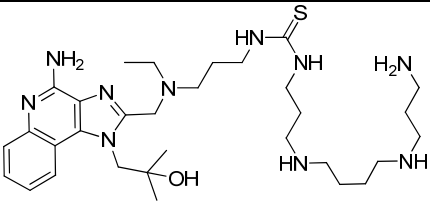
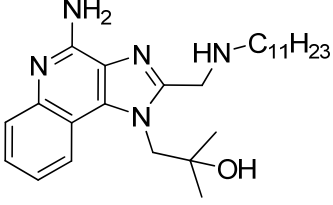
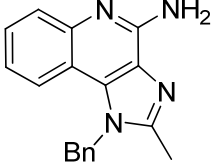
Given that TLR7 is sequestered in an acidic endolysosomal compartment, and these compounds are weak bases and are therefore expected to accumulate in that acidic organelle, we wondered if the observed SAR could simply be a consequence of differential hydrophobicity of the compounds, modulating uptake into the cell and entry into the endolysosomal compartment activity, rather than governing specific molecular interactions with TLR7. A comparison of observed retention times on C<sub>18</sub>-reverse-phase mass-chromatograms (a measure of hydrophobicity)<sup>169;170</sup> with the potencies of the active compounds indicate a distinct deviation from the trend of **54**, suggesting specific and idiosyncratic effects, rather than bulk-effects (**Fig. 5**).

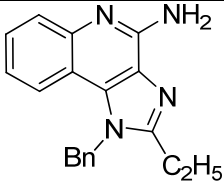
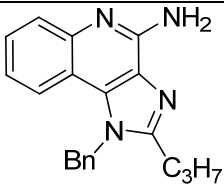
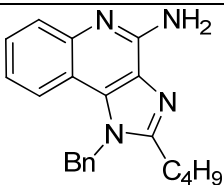
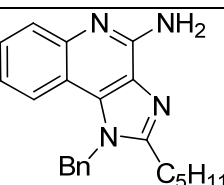
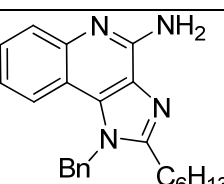
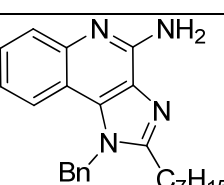
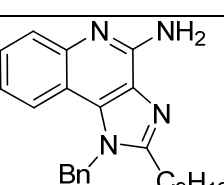
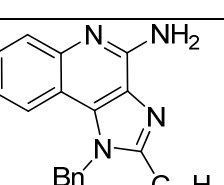
**Fig. 5.** Correlation of TLR7-agonistic potency and hydrophobicity (measured as retention time on  $C_{18}$  reverse-phase chromatography).



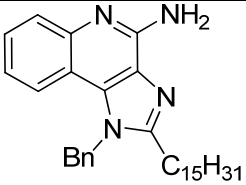
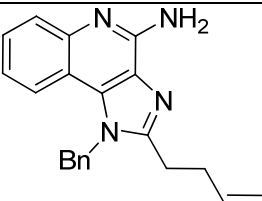
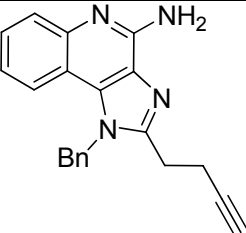
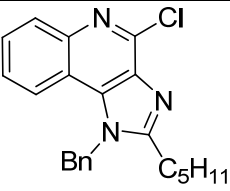
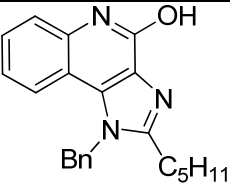
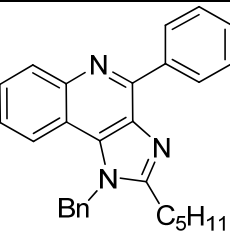
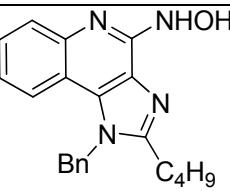
**Table 1.**  $EC_{50}$  values ( $\mu M$ ) of compounds in human TLR7-specific reporter gene assay. ND denotes no activity detected at 100  $\mu M$ .

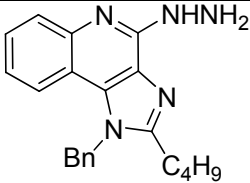
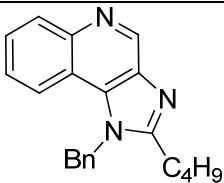
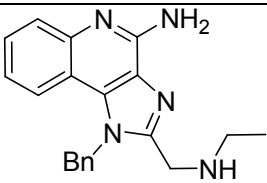
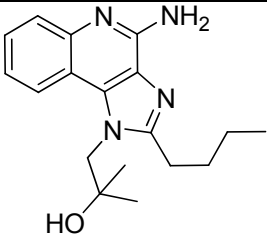
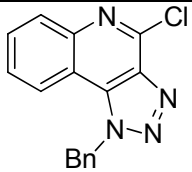
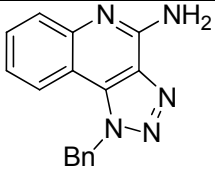
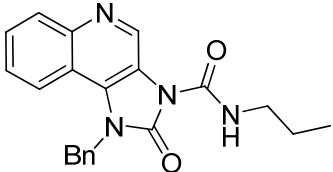
Structure	Compound Number	TLR7-Agonistic Activity ( $\mu M$ )
	1	2.12
	2	2.0
	3	7.71
	4	ND
	5	ND

	6	ND
	11	ND
	12	8.39
	13	ND
	16	ND
	22	ND
	28	0.955

	29	0.472
	30	0.647
	31	0.0591
	32	0.0915
	33	0.479
	34	0.822
	35	3.35
	36	ND



	37	ND
	38	0.262
	43	0.209
	44	ND
	46	ND
	47	ND
	48	0.723

	<b>49</b>	0.873
	<b>50</b>	ND
	<b>52</b>	0.385
	<b>54</b>	0.00858
	<b>55</b>	ND
	<b>56</b>	ND
	<b>57</b>	ND

### 4.3. Conclusions.

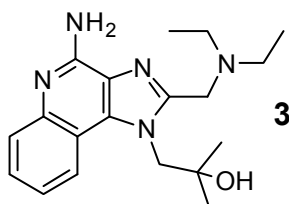
A detailed structure-activity study on the imidazoquinoline chemotype has been undertaken, which have been instructive and rewarding in that they not only complement the seminal work at 3M, but also have led to the discovery of highly-potent, lipophilic, human TLR7 agonist, **54**.

We note, not without irony, that this highly potent compound differs from its parent molecule by only a replacement of a secondary amine by a methylene unit. The oral bioavailability of **54** is predicted to be good, and it is possible that the systemic IFN- $\alpha$  inducing effects of this imidazoquinoline may find additional uses in the treatment of conditions such as hepatitis,<sup>171</sup> chronic myelogenous<sup>172</sup> and hairy cell leukemias.<sup>173</sup> However, for purposes of developing dermal vaccine adjuvants, **31** may be more efficacious for reasons discussed earlier.

#### 4.4. Experimental.

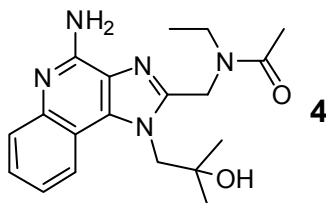
All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm x 150 mm, 5  $\mu$ m analytical reverse phase C<sub>18</sub> column with H<sub>2</sub>O-isopropanol or H<sub>2</sub>O-CH<sub>3</sub>CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion (or negative ion, as appropriate) acquisition mode. Unless otherwise mentioned, the compounds synthesized were obtained as solids.

**Synthesis of Compound 3: 1-(4-Amino-2-((diethylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol.**



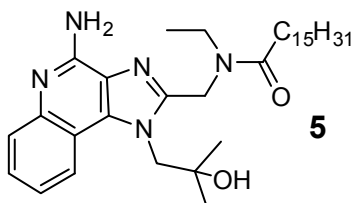
To a solution of trifluoroacetate salt of **2** (75 mg, 0.14 mmol) in anhydrous MeOH, were added acetaldehyde (6.6 mg, 0.15 mmol), 12-14 drops of acetic acid and sodium cyanoborohydride (10 mg, 0.15 mmol). After 4 hours the solvent was removed using vacuum and the residue was purified using column chromatography (7% MeOH/dichloromethane) to obtain the compound **3** (16 mg, 34%). <sup>1</sup>H NMR (500 MHz, MeOD) δ 8.36 (d, *J* = 7.8 Hz, 1H), 7.73 (dd, *J* = 8.4, 1.0 Hz, 1H), 7.69 – 7.64 (m, 1H), 7.52 (ddd, *J* = 8.4, 7.2, 1.2 Hz, 1H), 4.83 (s, 2H), 4.75 – 4.63 (m, 2H), 3.42 (dd, *J* = 14.2, 7.0 Hz, 4H), 1.39 – 1.29 (m, 6H), 1.21 (dd, *J* = 13.5, 6.2 Hz, 6H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 150.85, 150.16, 138.41, 136.09, 131.52, 126.42, 126.18, 123.69, 120.11, 114.65, 72.54, 56.68, 49.70, 49.56, 27.65, 9.45. MS (ESI) calculated for C<sub>19</sub>H<sub>27</sub>N<sub>5</sub>O, *m/z* 341.22, found 342.24 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 4: *N*-((4-Amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)-*N*-ethylacetamide.**



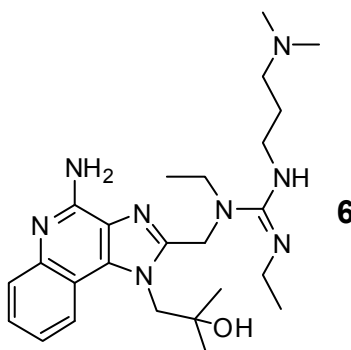
To a solution of trifluoroacetate salt of **2** (40 mg, 0.074 mmol) in anhydrous THF, were added triethylamine (23 mg, 0.22 mmol) and acetyl chloride (6 mg, 0.08 mmol). After 30 minutes, the solvent was removed under vacuum. The residue was dissolved in EtOAc, washed with water and dried over Na<sub>2</sub>SO<sub>4</sub>. The EtOAc fraction was then concentrated under vacuum and the residue was purified using column chromatography (6% MeOH/dichloromethane) to obtain the compound **4** as colorless oil (22 mg, 85%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.34 (d, *J* = 8.3 Hz, 1H), 7.71 (d, *J* = 8.3 Hz, 1H), 7.53 (t, *J* = 7.7 Hz, 1H), 7.38 (t, *J* = 7.7 Hz, 1H), 5.23 (s, 2H), 4.81 (s, 2H), 3.63 (q, *J* = 7.1 Hz, 2H), 2.23 (s, 3H), 1.34 – 1.13 (m, 9H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 172.18, 154.62, 136.88, 134.07, 129.68, 129.48, 124.60, 122.64, 122.31, 118.21, 113.67, 70.85, 65.49, 55.12, 43.70, 41.79, 19.68, 14.03, 12.30. MS (ESI) calculated for C<sub>19</sub>H<sub>25</sub>N<sub>5</sub>O<sub>2</sub>, *m/z* 355.20, found 356.21 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 5: *N*-((4-Amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)-*N*-ethylpalmitamide.**



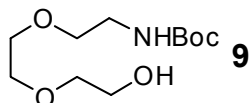
To a solution of trifluoroacetate salt of **2** (20 mg, 0.05 mmol) in anhydrous THF, were added triethylamine (15 mg, 0.15 mmol) and palmitoyl chloride (16 mg, 0.06 mmol). After 30 minutes the solvent was removed under vacuum, residue was dissolved in EtOAc and washed with water. The EtOAc fraction was then dried under vacuum and the residue was purified using column chromatography (5% MeOH/dichloromethane) to obtain the compound **5** as colorless oil (10 mg, 30%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.12 (d, *J* = 8.1 Hz, 1H), 7.88 (d, *J* = 8.3 Hz, 1H), 7.54 (t, *J* = 7.6 Hz, 1H), 7.38 (t, *J* = 7.8 Hz, 1H), 4.75 (s, 2H), 3.56 (q, *J* = 6.8 Hz, 2H), 2.45 – 2.34 (m, 2H), 1.76 – 1.57 (m, 2H), 1.58 – 1.11 (m, 35H), 0.90 (t, *J* = 6.8 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 192.82, 173.95, 152.03, 150.36, 135.45, 130.90, 128.29, 124.10, 123.24, 120.62, 114.81, 71.96, 55.58, 42.81, 42.30, 33.06, 31.92, 29.68, 29.65, 29.52, 29.47, 29.42, 29.35, 25.37, 22.69, 14.12, 13.70, 5.61. MS (ESI) calculated for C<sub>33</sub>H<sub>53</sub>N<sub>5</sub>O<sub>2</sub>, *m/z* 551.42, found 552.41 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 6: 1-((4-Amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)-3-(3-(dimethylamino)propyl)-1,2-diethylguanidine.**



To a solution of trifluoroacetate salt of **2** (50mg, 0.092 mmol) in anhydrous DMF, were added triethylamine (51 mg, 0.51 mmol), EDCI.HCl (53 mg, 0.28 mmol) and a catalytic amount of DMAP. The reaction was stirred for 24-30 hours. The solvent was then removed under vacuum, and the residue was purified using C<sub>18</sub> reverse-phase column chromatography to obtain the compound **6** (9 mg, 24%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.48 (d, *J* = 8.4 Hz, 1H), 7.84 (d, *J* = 7.4 Hz, 1H), 7.76 (t, *J* = 7.7 Hz, 1H), 7.63 (t, *J* = 7.2 Hz, 1H), 5.18 (s, 2H), 4.85 – 4.71 (m, 2H), 3.58 (q, *J* = 7.3 Hz, 2H), 3.54 – 3.41 (m, 4H), 3.27 – 3.18 (m, 2H), 2.90 (d, *J* = 3.7 Hz, 6H), 2.27 – 2.00 (m, 2H), 1.30 (dt, *J* = 14.4, 7.2 Hz, 12H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 160.10, 153.79, 137.08, 134.45, 129.86, 124.93, 122.19, 118.63, 113.38, 71.07, 55.30, 54.69, 46.25, 45.16, 42.06, 41.41, 39.78, 24.50, 13.79, 11.77. MS (ESI) calculated for C<sub>25</sub>H<sub>40</sub>N<sub>8</sub>O, *m/z* 468.33, found 469.33 (*M* + *H*)<sup>+</sup> and 235.17 (*M* + 2*H*)<sup>2+</sup>.

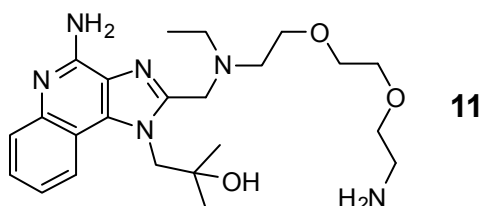
**Synthesis of Compound 9: *tert*-Butyl 2-(2-(2-hydroxyethoxy)ethoxy)ethylcarbamate.**



To a solution of **7** (500 mg, 2.1 mmol) in anhydrous dichloromethane, cooled to 0 °C, were added triethylamine (420 mg, 4.2 mmol), tosyl chloride (609 mg, 3.54 mmol) and a catalytic amount of DMAP. The reaction was stirred for 16 hours and then the mixture was washed with water and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was then removed under vacuum. This residue O-tosylated compound was dissolved in anhydrous DMF, to which sodium azide (273 mg, 4.2 mmol) was added and the reaction was heated at 70 °C for 1 hour. The solvent was then removed under vacuum and the residue was dissolved in EtOAc, washed with water and dried over Na<sub>2</sub>SO<sub>4</sub>. The EtOAc fraction was concentrated under vacuum to obtain the compound **8** (600mg). This **8** was then dissolved in MeOH and di-*tert*-butyl dicarbonate (687 mg, 3.5 mmol) was added to it. The solution was subjected to catalytic hydrogenolysis using Pd(OH)<sub>2</sub>/C at 60 psi hydrogen pressure for 4 hours. The solution was then filtered through celite and filtrate was evaporated under reduced pressure to afford the residue, which was purified using column chromatography (3% MeOH/dichloromethane) to obtain the

compound **9** as colorless oil (320 mg, 62%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  5.13 (s, 1H), 3.77 (d,  $J$  = 3.5 Hz, 2H), 3.70 – 3.61 (m, 6H), 3.58 (t,  $J$  = 5.2 Hz, 2H), 3.34 (d,  $J$  = 4.6 Hz, 2H), 2.50 (s, 1H), 1.46 (s, 9H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  159.62, 79.34, 72.55, 70.43, 70.29, 61.78, 40.34, 28.41. MS (ESI) calculated for  $\text{C}_{11}\text{H}_{23}\text{NO}_5$ ,  $m/z$  249.16, found 272.15 ( $\text{M} + \text{Na}^+$ ).

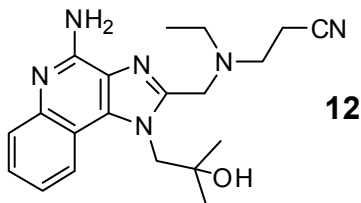
**Synthesis of Compound 11: 1-(4-Amino-2-(((2-(2-(2-aminoethoxy)ethoxy)ethyl)(ethyl)amino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol.**



To a solution of **9** (200mg, 0.8 mmol) in 4 mL of 0.3M Dess Martin periodinane solution in dichloromethane, 10-12 drops of acetic acid were added and the reaction was stirred for 4-5 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (40-50% EtOAc/dichloromethane) to obtain the intermediate aldehyde derivative **10** (71 mg). To a solution of trifluoroacetate salt of **2** (150 mg, 0.29 mmol) in anhydrous MeOH, were added **10** (71 mg, 0.29 mmol), 6-8 drops of acetic acid, and sodium cyanoborohydride (22 mg, 0.35 mmol). The solution was stirred for 14-15 hours and the solvent was removed under vacuum to obtain the residue, which was purified using column chromatography (10% MeOH/dichloromethane) to obtain the intermediate *N*-Boc derivative. This was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes, followed by removal of the solvent by purging nitrogen and drying under vacuum to obtain the trifluoroacetate salt of the compound **11** as colorless oil (110 mg, 60%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  8.49 (d,  $J$  = 8.4 Hz, 1H), 7.84 (d,  $J$  = 7.4 Hz, 1H), 7.76 (t,  $J$  = 7.3 Hz, 1H), 7.63 (t,  $J$  = 7.2 Hz, 1H), 4.82 (s, 4H), 3.88 (t,  $J$  = 5.1 Hz, 2H), 3.70 (d,  $J$  = 6.5 Hz, 6H), 3.50 (s, 2H), 3.38 (s, 2H), 3.17 – 3.09 (m, 2H), 1.55 – 1.10 (m, 9H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  149.53, 148.21, 136.86, 134.61, 130.12, 122.23, 118.64, 113.15, 71.15, 69.99, 69.93, 66.50, 64.83, 55.36, 52.60,

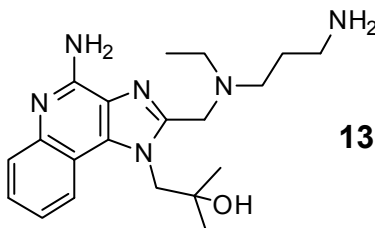
49.74, 48.69, 46.46, 39.10, 26.24, 7.96. MS (ESI) calculated for  $C_{23}H_{36}N_6O_3$ ,  $m/z$  444.29, found 467.27 ( $M + Na^+$ ) and 223.15 ( $M + 2H$ ) $^{2+}$ .

**Synthesis of Compound 12:** 3-(((4-Amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)(ethyl)amino)propanenitrile.



To a solution of **2** (250 mg, 0.46 mmol) in anhydrous MeOH, were added triethylamine (117 mg, 1.16 mmol) and acrylonitrile (61 mg, 1.16 mmol). The reaction was stirred for 30 hours, followed by removal of the solvent under vacuum. The residue was dissolved in EtOAc, washed with water and dried over  $Na_2SO_4$ . The EtOAc fraction was evaporated under vacuum and the residue was purified using column chromatography (11% MeOH/dichloromethane) to obtain the compound **12** (145 mg, 86%).  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.06 (d,  $J$  = 8.4 Hz, 1H), 7.87 (d,  $J$  = 8.3 Hz, 1H), 7.55 (t,  $J$  = 7.6 Hz, 1H), 7.36 (t,  $J$  = 7.7 Hz, 1H), 6.04 (s, 2H), 4.88 (s, 2H), 4.12 (s, 2H), 2.94 (t,  $J$  = 6.7 Hz, 2H), 2.75 (q,  $J$  = 7.0 Hz, 2H), 2.54 (t,  $J$  = 6.7 Hz, 2H), 1.35 (s, 6H), 1.19 (t,  $J$  = 7.2 Hz, 3H).  $^{13}C$  NMR (101 MHz,  $CDCl_3$ )  $\delta$  150.98, 150.41, 135.44, 127.81, 126.16, 122.48, 122.40, 122.18, 120.20, 118.64, 115.30, 71.49, 56.48, 50.85, 47.92, 47.64, 27.99, 15.33, 11.12. MS (ESI) calculated for  $C_{20}H_{26}N_6O$ ,  $m/z$  366.22, found 367.22 ( $M + H$ ) $^+$ .

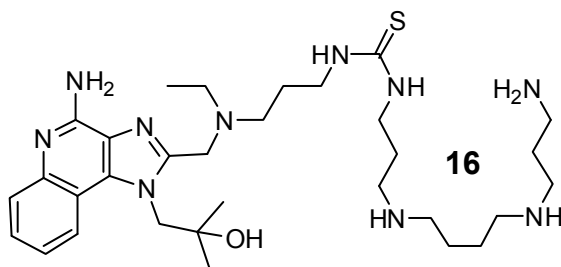
**Synthesis of Compound 13:** 1-(4-Amino-2-(((3-aminopropyl)(ethyl)amino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol.





To a solution of **12** (160 mg, 0.44 mmol) in glacial acetic acid, a catalytic amount of Pd(OH)<sub>2</sub>/C was added and the reaction mixture was subjected to hydrogenation at 55 psi hydrogen pressure for 4 hours. The solution was then filtered. The filtrate was concentrated to obtain the acetate salt of the compound **13** (55 mg). This residue (20 mg, 0.05 mmol) was then dissolved in MeOH and di-*tert*-butyl dicarbonate (18 mg, 0.08 mmol) was added. After 30 minutes, the solvent was removed under vacuum and the residue was purified using column chromatography (15% MeOH/dichloromethane) to obtain the intermediate *N*-Boc derivative. This was then dissolved in 2 mL of HCl solution in dioxane and stirred for 2 hours. The solvent was then removed under vacuum to obtain the hydrochloride salt of the compound **13** (15 mg, 74%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.51 (d, *J* = 8.2 Hz, 1H), 7.85 (d, *J* = 8.3 Hz, 1H), 7.78 (t, *J* = 7.9 Hz, 1H), 7.65 (t, *J* = 7.7 Hz, 1H), 5.09 (s, 2H), 3.74 – 3.50 (m, 6H), 3.12 (d, *J* = 7.0 Hz, 2H), 2.32 (s, 2H), 1.51 (t, *J* = 6.9 Hz, 3H), 1.35 (s, 6H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 156.18, 151.90, 140.30, 137.56, 129.79, 126.47, 124.63, 123.13, 123.00, 72.46, 56.49, 53.02, 52.27, 48.77, 40.46, 27.84, 24.89, 11.74. MS (ESI) calculated for C<sub>20</sub>H<sub>30</sub>N<sub>6</sub>O, *m/z* 370.25, found 371.26 (*M* + H)<sup>+</sup>.

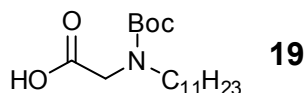
**Synthesis of Compound 16:** 1-(3-(((4-amino-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-2-yl)methyl)(ethyl)amino)propyl)-3-(3-(4-(3-aminopropylamino)butylamino)propyl)thiourea.



To a solution of **13** (35 mg, 0.07 mmol) in anhydrous ethanol, were added triethylamine (22 mg, 0.21 mmol) and CS<sub>2</sub> (54 mg, 0.71 mmol). The reaction mixture was stirred for 30 minutes and then cooled to 0 °C. Di-*tert*-butyl dicarbonate (15 mg, 0.07 mmol) and a catalytic amount of DMAP dissolved in ethanol were added to the reaction mixture. After 30 minutes, the solvent was removed under vacuum and the residue was purified using column chromatography to obtain the compound **15**. To a

solution of **15** (10mg, 0.02 mmol) in anhydrous dichloromethane,  $N^1, N^5, N^{10}$ -tris Boc spermine (37 mg, 0.073 mmol) was added. The reaction mixture was stirred for 3 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (6% MeOH/dichloromethane) to obtain the intermediate *N*-Boc derivative. This was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes, followed by removal of the solvent by purging nitrogen and drying under vacuum to obtain the trifluoroacetate salt of the compound **16** (5 mg, 20%).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.45 (d,  $J$  = 8.5 Hz, 1H), 7.83 (d,  $J$  = 8.4 Hz, 1H), 7.76 (t,  $J$  = 7.8 Hz, 1H), 7.67 – 7.56 (m, 1H), 4.96 (d,  $J$  = 6.9 Hz, 2H), 4.81 – 4.70 (m, 2H), 3.77 – 3.38 (m, 8H), 3.13 (dd,  $J$  = 18.0, 10.2 Hz, 2H), 3.10 – 2.96 (m, 8H), 2.16 – 2.04 (m, 4H), 1.97 – 1.88 (m, 2H), 1.84 – 1.74 (m, 4H), 1.44 (t,  $J$  = 7.2 Hz, 3H), 1.29 (s, 6H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  150.81, 138.54, 138.41, 135.99, 131.60, 126.50, 123.71, 120.06, 114.65, 72.55, 56.76, 52.24, 50.71, 49.86, 49.68, 48.26, 48.06, 46.25, 45.87, 37.80, 27.48, 25.39, 24.29, 9.47. MS (ESI) calculated for  $\text{C}_{31}\text{H}_{54}\text{N}_{10}\text{O}_5$ ,  $m/z$  614.42, found 615.43 ( $M + \text{H}$ ) $^+$  and 308.22 ( $M + 2\text{H}$ ) $^{2+}$ .

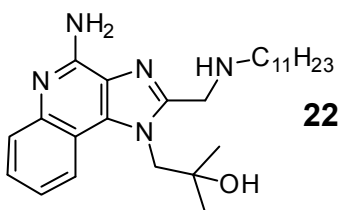
#### Synthesis of Compound 19: 2-(*tert*-Butoxycarbonyl(undecyl)amino)acetic acid.<sup>137</sup>



To a solution of **17** (200 mg, 1.6 mmol) in anhydrous MeOH, were added undecanal (218 mg, 1.28 mmol), 5-6 drops of acetic acid and sodium cyanoborohydride (111 mg, 1.76 mmol). The reaction was stirred for 3 hours and then the solvent was removed under vacuum. The residue was dissolved in dichloromethane and washed with water, brine and dried over  $\text{Na}_2\text{SO}_4$ . The dichloromethane fraction was then concentrated to obtain the residue **18**, which was dissolved in MeOH, followed by the addition of triethylamine (202 mg, 2 mmol) and di-*tert*-butyl dicarbonate (436 mg, 2 mmol). The reaction was stirred for 4 hours and the solvent was removed under vacuum. The residue was then dissolved in a solvent mixture of THF:MeOH (3:1) and lithium hydroxide that was dissolved in water was added until the pH went above 12. The reaction was stirred for 24 hours, followed by removal of the solvent under vacuum. The residue was dissolved in water and the solution was acidified using

10% HCl until pH was less than 2. The water fraction was then extracted with EtOAc, followed by washing the EtOAc fraction with water and drying over Na<sub>2</sub>SO<sub>4</sub>. The EtOAc fraction was then concentrated under vacuum to obtain the compound **19** as colorless oil (320 mg, 61%). MS (ESI) calculated for C<sub>18</sub>H<sub>35</sub>NO<sub>4</sub>, m/z 329.26, found 328.25 (M - H)<sup>-</sup>.

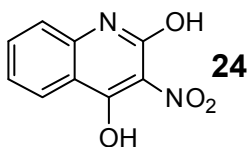
**Synthesis of Compound 22: 1-(4-Amino-2-((undecylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol.**



To a solution of **19** (171 mg, 0.52 mmol) in anhydrous DMF, were added HATU (217 mg, 0.57 mmol), triethylamine (58 mg, 0.57 mmol), a catalytic amount of DMAP and **20** (120 mg, 0.52 mmol). The reaction mixture was stirred for 3-4 hours. The solvent was evaporated under vacuum. The residue was dissolved in EtOAc, washed with water and dried over Na<sub>2</sub>SO<sub>4</sub> to obtain the residue. This was dissolved in 10 mL of ethanol and a solution of NaOH (105 mg, 2.62 mmol) dissolved in 1 mL of water was added to it. The reaction mixture was refluxed for 5-6 hours, followed by evaporation under reduced pressure. The residue was purified by column chromatography (5% MeOH/dichloromethane) to obtain the compound **21** (165 mg). To a solution of **21** in a solvent mixture of MeOH:dichloromethane:chloroform (0.1:1:1), 3-chloroperoxybenzoic acid (135 mg, 0.79 mmol) was added and the solution was refluxed at 45-50 °C for 40 minutes. The solvent was then removed and the residue was purified using column chromatography (7% MeOH/dichloromethane) to obtain the *N*-oxide derivative (75 mg). This was then dissolved in anhydrous dichloromethane, followed by the addition of benzoyl isocyanate (37 mg, 0.25 mmol) and heated at 45 °C for 15 minutes. The solvent was then removed under vacuum and the residue was dissolved in anhydrous MeOH, followed by the addition of excess sodium methoxide and heating at 80 °C for 1 hour. The solvent was then removed under vacuum and the residue was purified using column chromatography

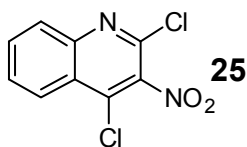
(8% MeOH/dichloromethane) to obtain the intermediate *N*-Boc derivative (27 mg). This was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes, followed by removal of the solvent by purging nitrogen and drying under vacuum to obtain the trifluoroacetate salt of the compound **22** as colorless oil (33 mg, 12%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 14.65 (s, 1H), 10.11 (s, 2H), 8.06 (s, 1H), 7.99 (d, *J* = 8.3 Hz, 1H), 7.44 (t, *J* = 7.6 Hz, 1H), 7.37 (t, *J* = 7.8 Hz, 1H), 7.20 (d, *J* = 8.6 Hz, 1H), 4.76 (s, 2H), 4.61 (s, 2H), 3.23 (s, 2H), 1.93 (s, 2H), 1.50 – 1.11 (m, 22H), 0.91 (t, *J* = 6.7 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 149.04, 148.91, 136.14, 134.18, 129.98, 124.99, 124.25, 120.76, 119.24, 112.18, 72.07, 49.07, 44.01, 31.89, 29.55, 29.49, 29.34, 29.31, 28.98, 26.58, 25.46, 22.67, 14.10, 5.61. MS (ESI) calculated for C<sub>26</sub>H<sub>41</sub>N<sub>5</sub>O, *m/z* 439.33, found 440.33 (*M* + *H*)<sup>+</sup> and 220.67 (*M* + 2*H*)<sup>2+</sup>.

#### Synthesis of Compound 24: 3-Nitroquinoline-2,4-diol.



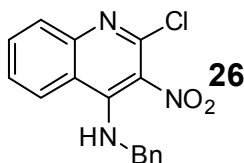
Compound **23** (5 g, 31 mmol) was dissolved in 30 mL of nitric acid and stirred at room temperature for 10 minutes, followed by heating the reaction mixture at 75 °C for another 15 minutes. The reaction mixture was then allowed to cool down to room temperature and was added to ice-water mixture to precipitate the product. The solid, yellow precipitate was filtered and dried to obtain the compound **24** (5.9 g, 92%). <sup>1</sup>H NMR (400 MHz, DMSO) δ 12.00 (s, 1H), 8.04 (d, *J* = 7.9 Hz, 1H), 7.66 (t, *J* = 7.5 Hz, 1H), 7.34 (d, *J* = 8.2 Hz, 1H), 7.28 (t, *J* = 7.7 Hz, 1H). <sup>13</sup>C NMR (101 MHz, DMSO) δ 156.69, 156.23, 138.56, 133.61, 127.71, 124.92, 122.81, 116.32, 114.47. MS (ESI) calculated for C<sub>9</sub>H<sub>6</sub>N<sub>2</sub>O<sub>4</sub>, *m/z* 206.03, found 205.04 (*M* - *H*)<sup>-</sup>.

#### Synthesis of Compound 25: 2,4-Dichloro-3-nitroquinoline.



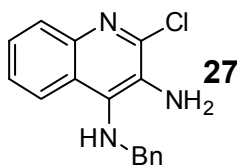
Compound **24** (2 g, 9.7 mmol) was dissolved in 20 mL of phenylphosphonyl dichloride and heated at 135 °C for 3 hours. The reaction mixture was poured into ice-water and stirred vigorously to obtain the precipitate, which was filtered and dried to afford the compound **25** (2.24 g, 74%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.30 (ddd, *J* = 8.4, 1.4, 0.6 Hz, 1H), 8.14 (ddd, *J* = 8.5, 1.2, 0.6 Hz, 1H), 7.97 (ddd, *J* = 8.5, 7.0, 1.4 Hz, 1H), 7.84 (ddd, *J* = 8.3, 7.0, 1.2 Hz, 1H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 146.64, 139.73, 135.62, 133.44, 129.76, 129.37, 125.22, 124.46.

#### Synthesis of Compound 26: *N*-Benzyl-2-chloro-3-nitroquinolin-4-amine.



To a solution of **25** (2.24 g, 9.26 mmol) in 30 mL of anhydrous dichloromethane, were added triethylamine (1.4 g, 13.9 mmol) and benzylamine (1.19 g, 11.1 mmol). The reaction mixture was refluxed at 45 °C for 30 minutes. The solvent was then evaporated under vacuum and water was added to the residue to obtain the precipitate. This was filtered, washed several times with water and dried to obtain the compound **26** (2.1 g, 73%). <sup>1</sup>H NMR (400 MHz, DMSO) δ 8.54 (dd, *J* = 12.9, 7.3 Hz, 2H), 7.89 – 7.81 (m, 2H), 7.69 (td, *J* = 8.6, 5.0 Hz, 1H), 7.39 – 7.21 (m, 5H), 4.46 (d, *J* = 6.3 Hz, 2H). <sup>13</sup>C NMR (101 MHz, DMSO) δ 145.79, 144.82, 141.51, 138.27, 132.70, 129.08, 128.94, 127.89, 127.40, 127.15, 126.96, 123.52, 120.12, 47.24. MS (ESI) calculated for C<sub>16</sub>H<sub>12</sub>ClN<sub>3</sub>O<sub>2</sub>, *m/z* 313.06, found 312.06 (*M* - H)<sup>-</sup>.

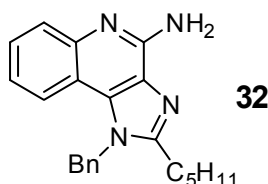
#### Synthesis of Compound 27: *N*<sup>4</sup>-Benzyl-2-chloroquinoline-3,4-diamine.



To a solution of **26** (2.21g, 7.06 mmol) in 20 mL of EtOAc, were added a catalytic amount of Pt/C and Na<sub>2</sub>SO<sub>4</sub>. The reaction mixture was subjected to hydrogenation at 55 psi hydrogen pressure for 3 hours. The reaction mixture was then filtered through celite and the filtrate was evaporated under vacuum to obtain the compound **27** (1.8 g, 90%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 7.90 (d, *J* = 8.2 Hz, 1H), 7.70 (d, *J* = 8.4 Hz, 1H), 7.43 (t, *J* = 7.6 Hz, 1H), 7.38 – 7.31 (m, 3H), 7.25 (t, *J* = 7.5 Hz, 2H), 7.20 (d, *J* = 7.0 Hz, 1H), 4.51 (s, 2H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 141.54, 141.28, 139.93, 137.86, 128.45, 128.04, 127.44, 126.96, 126.80, 126.22, 125.31, 123.22, 121.40, 49.56. MS (ESI) calculated for C<sub>16</sub>H<sub>14</sub>ClN<sub>3</sub>, *m/z* 283.09, found 284.10 (*M* + H)<sup>+</sup>.

#### General Procedure for Synthesis of C-2 alkyl imidazoquinoline compounds (28-38).

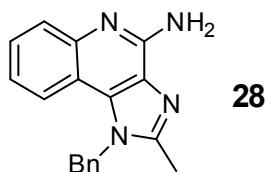
##### Synthesis of Compound 32: 1-Benzyl-2-pentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.



To a solution of **27** (50 mg, 0.18 mmol) in anhydrous THF, were added triethylamine (26 mg, 0.19 mmol) and hexanoyl chloride. The reaction mixture was stirred at room temperature for 6 hours. The solvent was then removed under vacuum, and the residue was dissolved in ethyl acetate and washed with water. The ethyl acetate fraction was dried using Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum to obtain the intermediate amide compound. This was dissolved in 0.1 mL of 2M solution of ammonia in MeOH. The sealed reaction vessel was heated in microwave at 150 °C for 1 hour. The solvent was then removed under vacuum and the residue was purified using column chromatography (7% MeOH/dichloromethane) to obtain the compound **32** (8 mg; unoptimized yields). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.81 (d, *J* = 8.3 Hz, 1H), 7.72 (dd, *J* = 8.3, 1.0 Hz, 1H), 7.44 (qd, *J* = 7.0, 3.5 Hz, 1H),

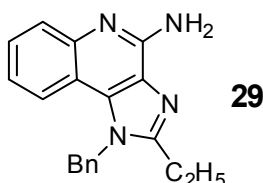
7.38 – 7.29 (m, 3H), 7.18 – 7.10 (m, 1H), 7.06 (d,  $J = 6.9$  Hz, 2H), 5.74 (s, 2H), 5.60 (s, 2H), 2.95 – 2.79 (m, 2H), 1.80 (dt,  $J = 15.6, 7.6$  Hz, 2H), 1.42 – 1.27 (m, 4H), 0.87 (t,  $J = 7.2$  Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  153.50, 150.28, 134.61, 133.41, 128.61, 127.40, 126.51, 125.94, 125.85, 124.85, 121.69, 119.07, 114.42, 48.20, 30.85, 26.97, 26.77, 21.65, 13.23. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{24}\text{N}_4$ ,  $m/z$  344.20, found 345.21 ( $\text{M} + \text{H}$ ) $^+$ . 2D  $^1\text{H}$  COSY and NOESY experiments (included in Supporting Information) provided unambiguous assignment of  $^1\text{H}$  resonances.

**28: 1-Benzyl-2-methyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.81 (dd,  $J = 8.4, 0.8$  Hz, 1H), 7.75 (dd,  $J = 8.3, 1.0$  Hz, 1H), 7.45 (ddd,  $J = 8.4, 7.1, 1.4$  Hz, 1H), 7.38 – 7.28 (m, 3H), 7.15 (ddd,  $J = 8.3, 7.1, 1.2$  Hz, 1H), 7.10 – 7.05 (m, 2H), 5.73 (s, 2H), 5.64 (s, 2H), 2.63 (s, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  149.17, 148.91, 142.26, 133.27, 132.64, 127.74, 126.56, 125.74, 124.90, 124.82, 123.96, 120.90, 117.99, 113.37, 47.59, 12.20. MS (ESI) calculated for  $\text{C}_{18}\text{H}_{16}\text{N}_4$ ,  $m/z$  288.14, found 289.15 ( $\text{M} + \text{H}$ ) $^+$ .

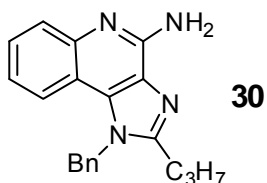
**29: 1-Benzyl-2-ethyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.80 (dd,  $J = 8.4, 0.8$  Hz, 1H), 7.73 (dd,  $J = 8.3, 1.0$  Hz, 1H), 7.44 (qd,  $J = 7.2, 3.7$  Hz, 1H), 7.37 – 7.28 (m, 3H), 7.13 (ddd,  $J = 8.3, 7.0, 1.3$  Hz, 1H), 7.09 – 7.01 (m, 2H), 5.73 (s, 2H), 5.59 (s, 2H), 2.97 – 2.86 (m, 2H), 1.41 (t,  $J = 7.5$  Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  153.83, 149.91, 143.24, 134.14, 133.05, 128.19, 126.96, 126.04, 125.64, 125.52, 124.43, 121.20,

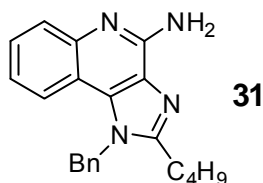
118.62, 114.05, 47.69, 19.72, 10.98. MS (ESI) calculated for  $C_{19}H_{18}N_4$ ,  $m/z$  302.15, found 303.16 ( $M + H$ )<sup>+</sup>.

**30: 1-Benzyl-2-propyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



<sup>1</sup>H NMR (500 MHz,  $CDCl_3$ )  $\delta$  7.80 (dd,  $J = 8.4, 0.8$  Hz, 1H), 7.71 (dd,  $J = 8.3, 1.0$  Hz, 1H), 7.49 – 7.42 (m, 1H), 7.40 – 7.30 (m, 3H), 7.18 – 7.10 (m, 1H), 7.10 – 7.05 (m, 2H), 5.74 (s, 2H), 5.66 (s, 2H), 2.92 – 2.80 (m, 2H), 1.91 – 1.85 (m, 2H), 1.03 (t,  $J = 7.4$  Hz, 3H). <sup>13</sup>C NMR (126 MHz,  $CDCl_3$ )  $\delta$  153.50, 150.46, 143.54, 134.77, 133.58, 128.82, 127.60, 126.73, 126.12, 126.05, 125.01, 121.90, 119.30, 114.59, 48.36, 28.88, 20.92, 13.52. MS (ESI) calculated for  $C_{20}H_{20}N_4$ ,  $m/z$  316.17, found 317.17 ( $M + H$ )<sup>+</sup>.

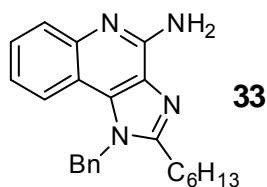
**31: 1-Benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



<sup>1</sup>H NMR (500 MHz,  $CDCl_3$ )  $\delta$  7.80 (dd,  $J = 8.4, 0.8$  Hz, 1H), 7.72 (dd,  $J = 8.3, 1.0$  Hz, 1H), 7.43 (ddd,  $J = 8.4, 7.0, 1.4$  Hz, 1H), 7.36 – 7.28 (m, 3H), 7.12 (ddd,  $J = 8.3, 7.1, 1.2$  Hz, 1H), 7.08 – 7.04 (m, 2H), 5.73 (s, 2H), 5.56 (s, 2H), 2.91 – 2.83 (m, 2H), 1.84 – 1.74 (m, 2H), 1.43 (dq,  $J = 14.8, 7.4$  Hz, 2H), 0.92 (t,  $J = 7.4$  Hz, 3H). <sup>13</sup>C NMR (126 MHz,  $CDCl_3$ )  $\delta$  152.56, 149.54, 142.97, 133.87, 132.54, 127.81, 126.58, 125.61, 125.34, 125.21, 124.06, 120.78, 118.25, 113.71, 47.38, 28.54, 25.71, 21.04, 12.28. MS (ESI) calculated for  $C_{21}H_{22}N_4$ ,  $m/z$  330.18, found 331.19 ( $M + H$ )<sup>+</sup>.

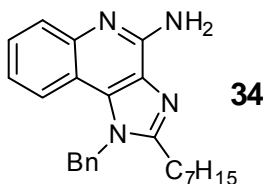


**33: 1-Benzyl-2-hexyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



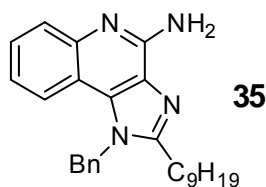
$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.82 (d,  $J$  = 8.4 Hz, 1H), 7.72 (d,  $J$  = 8.2 Hz, 1H), 7.45 (t,  $J$  = 7.7 Hz, 1H), 7.38 – 7.29 (m, 3H), 7.13 (t,  $J$  = 7.6 Hz, 1H), 7.07 (d,  $J$  = 7.4 Hz, 2H), 5.73 (s, 2H), 5.63 (s, 2H), 2.93 – 2.83 (m, 2H), 1.87 – 1.73 (m, 2H), 1.40 (dd,  $J$  = 14.3, 6.7 Hz, 2H), 1.34 – 1.24 (m, 4H), 0.88 (t,  $J$  = 6.5 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  154.01, 151.10, 144.59, 135.38, 133.98, 129.25, 128.02, 127.02, 126.87, 126.69, 125.54, 122.17, 119.70, 115.20, 48.84, 31.44, 29.05, 27.90, 27.46, 22.46, 14.01. MS (ESI) calculated for  $\text{C}_{23}\text{H}_{26}\text{N}_4$ ,  $m/z$  358.22, found 359.22 ( $\text{M} + \text{H}$ ) $^+$ .

**34: 1-Benzyl-2-heptyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



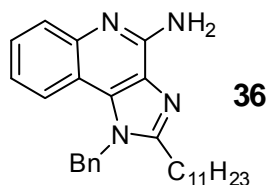
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.81 (dd,  $J$  = 8.4, 0.8 Hz, 1H), 7.71 (dd,  $J$  = 8.3, 1.0 Hz, 1H), 7.44 (ddd,  $J$  = 8.4, 7.1, 1.3 Hz, 1H), 7.35 – 7.29 (m, 3H), 7.12 (ddd,  $J$  = 8.2, 7.1, 1.2 Hz, 1H), 7.05 (d,  $J$  = 6.9 Hz, 2H), 5.72 (s, 4H), 2.86 (dd,  $J$  = 18.2, 10.2 Hz, 2H), 1.79 (dt,  $J$  = 15.5, 7.7 Hz, 2H), 1.39 (dt,  $J$  = 14.9, 7.0 Hz, 2H), 1.34 – 1.18 (m, 6H), 0.86 (t,  $J$  = 7.0 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  152.52, 149.22, 142.11, 133.55, 132.39, 127.57, 126.36, 125.49, 124.86, 124.69, 123.80, 120.69, 118.05, 113.31, 47.15, 29.89, 27.60, 27.19, 26.21, 25.75, 20.85, 12.34. MS (ESI) calculated for  $\text{C}_{24}\text{H}_{28}\text{N}_4$ ,  $m/z$  372.23, found 373.24 ( $\text{M} + \text{H}$ ) $^+$ .

**35: 1-Benzyl-2-nonyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



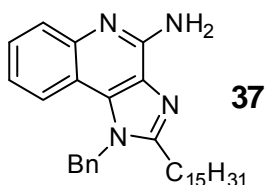
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.85 – 7.79 (m, 1H), 7.71 (dd,  $J$  = 8.3, 0.9 Hz, 1H), 7.44 (qd,  $J$  = 6.9, 3.4 Hz, 1H), 7.38 – 7.29 (m, 3H), 7.13 (ddd,  $J$  = 8.2, 7.1, 1.2 Hz, 1H), 7.05 (d,  $J$  = 6.9 Hz, 2H), 5.85 (s, 2H), 5.72 (s, 2H), 2.90 – 2.84 (m, 2H), 1.87 – 1.71 (m, 2H), 1.44 – 1.33 (m, 2H), 1.33 – 1.18 (m, 10H), 0.87 (t,  $J$  = 7.0 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  152.31, 148.72, 141.13, 133.07, 132.10, 127.20, 126.00, 125.25, 124.35, 123.92, 123.40, 120.47, 117.71, 112.77, 46.79, 29.72, 27.59, 27.26, 27.24, 27.13, 27.12, 25.78, 25.35, 20.53, 12.00. MS (ESI) calculated for  $\text{C}_{26}\text{H}_{32}\text{N}_4$ ,  $m/z$  400.26, found 401.27 ( $\text{M} + \text{H}$ ) $^+$ .

**36: 1-Benzyl-2-undecyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



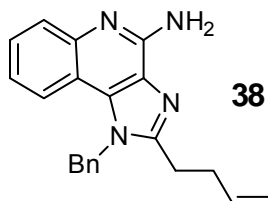
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.81 (d,  $J$  = 8.2 Hz, 1H), 7.71 (d,  $J$  = 7.7 Hz, 1H), 7.47 – 7.42 (m, 1H), 7.36 – 7.28 (m, 3H), 7.15 – 7.11 (m, 1H), 7.06 (d,  $J$  = 7.0 Hz, 2H), 5.73 (s, 2H), 5.70 (s, 2H), 3.02 – 2.71 (m, 2H), 1.79 (dt,  $J$  = 15.5, 7.8 Hz, 2H), 1.44 – 1.34 (m, 2H), 1.34 – 1.18 (m, 14H), 0.88 (t,  $J$  = 7.0 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  152.56, 149.23, 142.11, 133.58, 132.43, 127.60, 126.39, 125.53, 124.89, 124.73, 123.83, 120.73, 118.08, 113.34, 47.19, 30.20, 27.88, 27.73, 27.67, 27.62, 27.56, 26.24, 25.78, 20.98, 12.43. MS (ESI) calculated for  $\text{C}_{28}\text{H}_{36}\text{N}_4$ ,  $m/z$  428.29, found 429.30 ( $\text{M} + \text{H}$ ) $^+$ .

**37: 1-Benzyl-2-pentadecyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



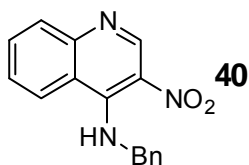
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.81 (dd,  $J = 8.4, 0.8$  Hz, 1H), 7.72 (dd,  $J = 8.3, 1.0$  Hz, 1H), 7.44 (ddd,  $J = 8.4, 7.1, 1.3$  Hz, 1H), 7.36 – 7.27 (m, 3H), 7.13 (ddd,  $J = 8.3, 7.1, 1.2$  Hz, 1H), 7.09 – 7.02 (m, 2H), 5.73 (s, 2H), 5.69 (s, 2H), 2.91 – 2.83 (m, 2H), 1.79 (dt,  $J = 15.5, 7.7$  Hz, 2H), 1.59 – 1.33 (m, 2H), 1.33 – 1.16 (m, 22H), 0.88 (t,  $J = 7.0$  Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  151.99, 148.66, 141.59, 133.01, 131.85, 127.03, 125.82, 124.96, 124.32, 124.17, 123.26, 120.16, 117.50, 112.78, 46.62, 29.66, 27.43, 27.40, 27.39, 27.37, 27.32, 27.17, 27.11, 27.10, 26.99, 25.68, 25.21, 20.43, 11.86. MS (ESI) calculated for  $\text{C}_{32}\text{H}_{44}\text{N}_4$ ,  $m/z$  484.36, found 485.36 ( $\text{M} + \text{H}$ ) $^+$ .

**38: 1-Benzyl-2-(but-3-enyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



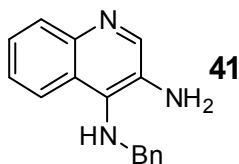
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.80 (dd,  $J = 8.4, 0.8$  Hz, 1H), 7.73 (dd,  $J = 8.3, 1.0$  Hz, 1H), 7.46 – 7.42 (m, 1H), 7.36 – 7.29 (m, 3H), 7.13 (ddd,  $J = 8.2, 7.1, 1.2$  Hz, 1H), 7.08 – 7.05 (m, 2H), 5.96 – 5.84 (m, 1H), 5.74 (s, 2H), 5.58 (s, 2H), 5.11 – 4.97 (m, 2H), 3.03 – 2.93 (m, 2H), 2.64 – 2.54 (m, 2H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  150.99, 148.87, 134.50, 133.06, 131.93, 127.16, 125.97, 125.02, 124.64, 124.58, 123.43, 120.17, 117.57, 113.92, 46.72, 29.59, 24.77. MS (ESI) calculated for  $\text{C}_{21}\text{H}_{20}\text{N}_4$ ,  $m/z$  328.17, found 329.18 ( $\text{M} + \text{H}$ ) $^+$ .

#### Synthesis of Compound 40: *N*-Benzyl-3-nitroquinolin-4-amine.



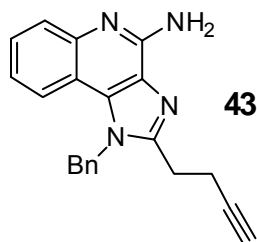
To a solution of **39** (2.03 mg, 9.76 mmol) in 20 mL of anhydrous dichloromethane, were added triethylamine (1.48 g, 14.64 mmol) and benzylamine (1.36 g, 12.69 mmol). The reaction mixture was refluxed at 45 °C for 30 minutes. The solvent was then evaporated under vacuum and water was added to the residue to obtain the precipitate. This was filtered, washed several times with water and dried to obtain the compound **40** (2.6 g, 95%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.89 (s, 1H), 9.42 (s, 1H), 8.31 (d, *J* = 8.6 Hz, 1H), 8.04 (d, *J* = 8.4 Hz, 1H), 7.79 (t, *J* = 7.6 Hz, 1H), 7.56 – 7.35 (m, 6H), 5.13 (d, *J* = 5.7 Hz, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 151.01, 150.64, 147.37, 136.84, 132.73, 130.51, 129.36, 128.54, 127.16, 126.84, 126.33, 125.58, 119.14, 53.06. MS (ESI) calculated for C<sub>16</sub>H<sub>13</sub>N<sub>3</sub>O<sub>2</sub>, *m/z* 279.10, found 280.11 (*M* + H)<sup>+</sup>.

#### Synthesis of Compound 41: *N*<sup>4</sup>-Benzylquinoline-3,4-diamine.



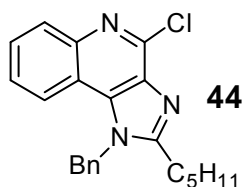
To a solution of **40** (2.6 g, 7.06 mmol) in 10 mL of EtOAc, were added a catalytic amount of Pt/C and Na<sub>2</sub>SO<sub>4</sub>. The reaction mixture was subjected to hydrogenation at 55 psi hydrogen pressure for 4 hours. The reaction mixture was then filtered through celite and the filtrate was evaporated under vacuum to obtain the compound **41** (2.3 g, 96%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.53 (s, 1H), 8.00 (d, *J* = 8.3 Hz, 1H), 7.75 (d, *J* = 8.2 Hz, 1H), 7.56 – 7.29 (m, 7H), 4.43 (s, 2H), 4.05 (s, 1H), 3.78 (s, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 143.59, 143.48, 141.10, 139.77, 129.89, 129.84, 128.81, 127.77, 127.60, 126.05, 125.76, 120.06, 99.98, 50.98. MS (ESI) calculated for C<sub>16</sub>H<sub>15</sub>N<sub>3</sub>, *m/z* 249.13, found 250.13 (*M* + H)<sup>+</sup>.

**Synthesis of Compound 43: 1-Benzyl-2-(but-3-ynyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



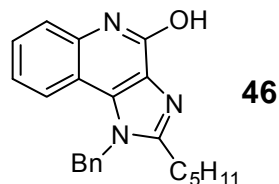
To a solution of pentynoic acid (37 mg, 0.38 mmol) in anhydrous DMF, were added HBTU (142 mg, 0.38 mmol), triethylamine (52 mg, 0.51 mmol), a catalytic amount of DMAP and **41** (85 mg, 0.34 mmol). The reaction mixture was then heated to 90 °C for 1 hour. The solvent was then removed under vacuum. The residue was dissolved in EtOAc, washed with water, dried over Na<sub>2</sub>SO<sub>4</sub> and then concentrated to obtain the residue. This was purified using column chromatography (7% MeOH/dichloromethane) to obtain the compound **42** (60 mg). To a solution of **42** in a solvent mixture of MeOH:dichloromethane:chloroform (0.1:1:1), was added 3-chloroperoxybenzoic acid (83 mg, 0.48 mmol), and the solution was refluxed at 45-50 °C for 40 minutes. The solvent was then removed under vacuum and the residue was purified using column chromatography (8% MeOH/dichloromethane) to obtain the *N*-oxide derivative (30 mg). This was then dissolved in anhydrous dichloromethane, followed by the addition of benzoyl isocyanate (20 mg, 0.14 mmol) and heated at 45 °C for 15 minutes. The solvent was then removed under vacuum and the residue was dissolved in anhydrous MeOH, followed by the addition of excess sodium methoxide. The reaction mixture was then heated at 80 °C for 1 hour. The solvent was removed under vacuum and the residue was purified using column chromatography (6% MeOH/dichloromethane) to obtain the compound **43** (15 mg, 12%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.81 (dd, *J* = 8.4, 0.8 Hz, 1H), 7.74 (dd, *J* = 8.3, 1.0 Hz, 1H), 7.46 (ddd, *J* = 8.4, 5.7, 1.3 Hz, 1H), 7.37 – 7.30 (m, 3H), 7.15 (ddd, *J* = 8.3, 7.1, 1.2 Hz, 1H), 7.09 – 7.05 (m, 2H), 5.79 (s, 2H), 5.68 (s, 2H), 3.11 (dd, *J* = 8.8, 6.4 Hz, 2H), 2.87 – 2.71 (m, 2H), 1.99 (t, *J* = 2.6 Hz, 1H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 150.49, 149.42, 133.45, 132.82, 127.91, 126.75, 126.01, 125.19, 124.98, 124.08, 121.13, 118.33, 113.52, 81.16, 68.15, 47.46, 25.06, 15.63. MS (ESI) calculated for C<sub>21</sub>H<sub>18</sub>N<sub>4</sub>, *m/z* 326.15, found 327.16 (*M* + H)<sup>+</sup>.

#### Synthesis of Compound 44: 1-Benzyl-4-chloro-2-pentyl-1*H*-imidazo[4,5-*c*]quinoline.



To a solution **27** (200 mg, 0.71 mmol) in anhydrous THF, were added triethylamine (179 mg, 1.77 mmol) and hexanoyl chloride (143 mg, 1.06 mmol). The reaction mixture was then stirred for 6-8 hours, followed by removal of the solvent under vacuum. The residue was dissolved in EtOAc, washed with water, brine and then dried over Na<sub>2</sub>SO<sub>4</sub> to obtain the intermediate amide compound. This was dissolved in MeOH, followed by the addition of calcium oxide. The reaction mixture was then heated in microwave at 110 °C for 1 hour. The solvent was removed under vacuum and the residue was purified using column chromatography (3% MeOH/dichloromethane) to obtain the compound **44** (170 mg, 66%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.16 (d, *J* = 8.4 Hz, 1H), 7.88 (d, *J* = 8.4 Hz, 1H), 7.60 (t, *J* = 7.7 Hz, 1H), 7.42 (t, *J* = 7.7 Hz, 1H), 7.38 – 7.30 (m, 3H), 7.05 (d, *J* = 7.0 Hz, 2H), 5.81 (s, 2H), 3.05 – 2.95 (m, 2H), 1.84 (dt, *J* = 15.6, 7.6 Hz, 2H), 1.48 – 1.24 (m, 4H), 0.88 (t, *J* = 7.1 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 156.40, 143.96, 143.60, 135.40, 134.74, 133.74, 129.93, 129.42, 128.30, 127.56, 126.58, 125.40, 119.91, 117.29, 49.19, 31.63, 27.91, 27.70, 22.29, 13.90. MS (ESI) calculated for C<sub>22</sub>H<sub>22</sub>ClN<sub>3</sub>, *m/z* 363.15, found 364.15 (*M* + *H*)<sup>+</sup>.

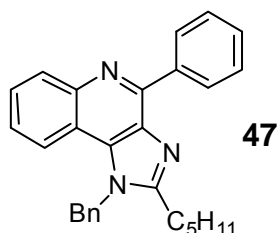
#### Synthesis of Compound 46: 1-Benzyl-2-pentyl-1*H*-imidazo[4,5-*c*]quinolin-4-ol.



The compound **44** (50 mg, 0.14 mmol) was dissolved in 6M HCl and the solution was heated in microwave at 110 °C for 1 hour. The solvent was then removed under vacuum and the residue was purified using column chromatography (7% MeOH/dichloromethane) to obtain the compound **46** (38

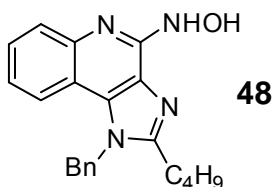
mg, 80%).  $^1\text{H}$  NMR (400 MHz, DMSO)  $\delta$  11.59 (s, 1H), 7.74 (d,  $J$  = 8.2 Hz, 1H), 7.43 (d,  $J$  = 8.2 Hz, 1H), 7.39 – 7.30 (m,  $J$  = 8.3 Hz, 3H), 7.30 – 7.23 (m,  $J$  = 7.2 Hz, 1H), 7.08 – 7.00 (m,  $J$  = 7.5 Hz, 3H), 5.83 (s, 2H), 2.83 (t,  $J$  = 7.5 Hz, 2H), 1.79 – 1.65 (m, 2H), 1.40 – 1.16 (m, 4H), 0.82 (t,  $J$  = 7.0 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, DMSO)  $\delta$  157.78, 154.36, 137.04, 136.73, 134.44, 131.09, 129.43, 128.08, 127.95, 125.94, 121.98, 121.27, 116.69, 112.04, 48.44, 31.22, 27.11, 26.69, 22.30, 14.26. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{23}\text{N}_3$ ,  $m/z$  345.18, found 346.19 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 47: 1-Benzyl-2-pentyl-4-phenyl-1*H*-imidazo[4,5-*c*]quinoline.**



Suzuki coupling of the compound **44** with phenyl boronic acid was performed. To a solution of **44** (70 mg, 0.19 mmol) in THF, were added polystyrene-bound  $\text{PPh}_3\text{-Pd}$  (9.5 mg, 0.00095 mmol),  $\text{K}_2\text{CO}_3$  (40 mg, 0.29 mmol) dissolved in water and phenyl boronic acid (28 mg, 0.23 mmol). The reaction was heated at 80  $^\circ\text{C}$  for 10-12 hours. The solution was filtered to remove the solid resin and the filtrate was evaporated under vacuum to obtain the residue. This was purified using column chromatography (30% EtOAc/hexane) to obtain the compound **47** (25 mg, 33%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.81 – 8.77 (m, 1H), 8.33 (dd,  $J$  = 8.5, 0.8 Hz, 1H), 7.93 (dd,  $J$  = 8.4, 0.8 Hz, 1H), 7.64 – 7.58 (m, 3H), 7.55 – 7.49 (m, 1H), 7.36 (dddd,  $J$  = 9.8, 6.9, 6.1, 2.2 Hz, 4H), 7.12 – 7.07 (m, 2H), 5.84 (s, 2H), 3.03 – 2.85 (m, 2H), 1.91 (dt,  $J$  = 15.5, 7.6 Hz, 2H), 1.42 (ddt,  $J$  = 36.4, 21.9, 7.4 Hz, 4H), 0.92 (t,  $J$  = 7.2 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  155.17, 151.02, 144.50, 138.18, 135.43, 135.10, 135.01, 130.96, 130.01, 129.31, 129.27, 128.37, 128.05, 126.74, 125.76, 125.52, 119.61, 117.17, 48.92, 31.58, 27.56, 27.48, 22.37, 13.97. MS (ESI) calculated for  $\text{C}_{28}\text{H}_{27}\text{N}_3$ ,  $m/z$  405.22, found 406.23 ( $\text{M} + \text{H}$ ) $^+$ .

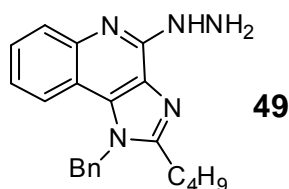
**Synthesis of Compound 48: *N*-(1-Benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-yl)hydroxylamine.**



To a solution of **27** (100 mg, 0.35 mmol) in anhydrous THF, were added triethylamine (72 mg, 0.71 mmol) and valeryl chloride (51 mg, 0.42 mmol). The reaction mixture was then stirred for 6-8 hours, followed by removal of the solvent under vacuum. The residue was dissolved in EtOAc, washed with water, brine and then dried over Na<sub>2</sub>SO<sub>4</sub> to obtain the residue. This was dissolved in MeOH, followed by the addition of calcium oxide. The reaction mixture was then heated in microwave at 110 °C for 1 hour. The solvent was removed under vacuum and the residue was purified using column chromatography (3% MeOH/dichloromethane) to obtain **45** (75 mg, 62%). To a solution of **45** (25 mg, 0.072 mmol) in anhydrous MeOH, were added triethylamine (72 mg, 0.72 mmol) and hydroxylamine hydrochloride (76 mg, 1.08 mmol). The reaction mixture was heated at 50 °C for 10-12 hours. The solvent was then removed under vacuum and the residue was dissolved in EtOAc, washed with water and dried over Na<sub>2</sub>SO<sub>4</sub>. The EtOAc fraction was then evaporated under vacuum and the residue was purified using basic-alumina (pH>7) column chromatography (30% MeOH/dichloromethane) to obtain the compound **48** (10 mg, 40%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.41 (d, *J* = 8.1 Hz, 1H), 7.32 (dq, *J* = 25.0, 7.2 Hz, 4H), 7.21 (s, 1H), 7.06 (d, *J* = 7.1 Hz, 2H), 6.88 (dd, *J* = 10.6, 5.7 Hz, 1H), 5.61 (s, 2H), 2.84 – 2.77 (m, 2H), 1.78 (dt, *J* = 15.5, 7.7 Hz, 2H), 1.40 (dt, *J* = 15.0, 7.4 Hz, 2H), 0.90 – 0.86 (m, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 151.78, 133.18, 127.13, 125.87, 125.13, 123.30, 119.10, 117.94, 46.50, 27.68, 24.81, 20.34, 11.58. MS (ESI) calculated for C<sub>21</sub>H<sub>22</sub>N<sub>4</sub>O, *m/z* 346.18, found 347.19 (*M* + *H*)<sup>+</sup>.

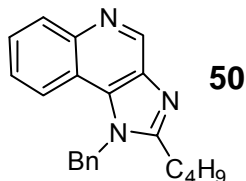


### Synthesis of Compound 49: 1-Benzyl-2-butyl-4-hydrazinyl-1*H*-imidazo[4,5-*c*]quinoline.



To a solution of **45** (60 mg, 0.17 mmol) in anhydrous MeOH, was added *tert*-butyl carbazate (57 mg, 0.43 mmol). After 15 hours, the solvent was removed under vacuum and the residue was purified using column chromatography (4% MeOH/dichloromethane) to obtain the intermediate *N*-Boc derivative. This was then dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes, followed by removal of the solvent by purging nitrogen and drying under vacuum to obtain the trifluoroacetate salt of the compound **49** (55 mg, 57%). <sup>1</sup>H NMR (500 MHz, MeOD)  $\delta$  7.82 (d, *J* = 8.4 Hz, 1H), 7.79 (d, *J* = 8.3 Hz, 1H), 7.45 – 7.40 (m, 1H), 7.28 (dt, *J* = 25.5, 7.2 Hz, 3H), 7.15 – 7.10 (m, 1H), 7.03 (d, *J* = 7.4 Hz, 2H), 5.82 (s, 2H), 2.94 – 2.90 (m, 2H), 1.79 – 1.69 (m, 2H), 1.40 (dq, *J* = 14.8, 7.4 Hz, 2H), 0.90 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (126 MHz, MeOD)  $\delta$  154.65, 151.25, 143.19, 135.64, 133.54, 128.81, 128.68, 127.41, 126.96, 125.31, 125.10, 124.68, 122.23, 120.02, 114.47, 48.23, 29.28, 26.26, 21.84, 12.51. MS (ESI) calculated for C<sub>21</sub>H<sub>23</sub>N<sub>5</sub>, *m/z* 345.20, found 346.20 (*M* + H)<sup>+</sup>.

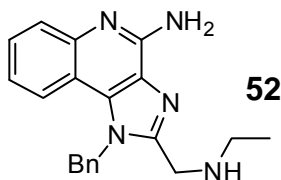
### Synthesis of Compound 50: 1-Benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinoline.



To a solution of **41** (300 mg, 1.221 mmol) in anhydrous THF, were added triethylamine (183 mg, 1.82 mmol) and valeryl chloride (174 mg, 1.45 mmol). The reaction mixture was then stirred for 6-8 hours, followed by removal of the solvent under vacuum. The residue was dissolved in EtOAc, washed with water, brine and then dried over Na<sub>2</sub>SO<sub>4</sub> to obtain the intermediate amide compound. This was

dissolved in MeOH, followed by the addition of calcium oxide. The reaction mixture was then heated in microwave at 110 °C for an hour. The solvent was removed under vacuum and the residue was purified using column chromatography (9% MeOH/dichloromethane) to obtain the compound **50** (360 mg, 94%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.38 (s, 1H), 8.25 (d, *J* = 8.1 Hz, 1H), 7.93 (d, *J* = 8.5 Hz, 1H), 7.66 – 7.57 (m, 1H), 7.42 (dd, *J* = 11.2, 4.1 Hz, 1H), 7.40 – 7.31 (m, 3H), 7.06 (d, *J* = 6.6 Hz, 2H), 5.80 (s, 2H), 3.02 – 2.85 (m, 2H), 1.90 (dt, *J* = 15.4, 7.7 Hz, 2H), 1.57 – 1.39 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 155.81, 144.98, 144.75, 136.53, 135.14, 134.10, 130.83, 129.35, 128.15, 126.72, 126.29, 125.47, 119.88, 117.58, 48.94, 29.57, 27.18, 22.53, 13.77. MS (ESI) calculated for C<sub>21</sub>H<sub>21</sub>N<sub>3</sub>, *m/z* 315.17, found 316.18 (*M* + H)<sup>+</sup>.

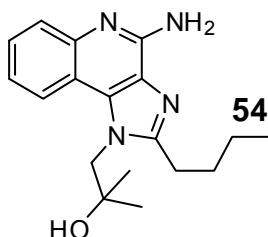
**Synthesis of Compound 52: 1-Benzyl-2-((ethylamino)methyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



To a solution of 2-(*tert*-butoxycarbonyl(ethyl)amino)acetic acid (90 mg, 0.44 mmol) in anhydrous DMF, were added HBTU (167 mg, 0.44 mmol), triethylamine (53 mg, 0.52 mmol), a catalytic amount of DMAP and **41** (100 mg, 0.4 mmol). The reaction mixture was then heated to 70 °C for 18 hours. The solvent was then removed under vacuum. The residue was dissolved in EtOAc, washed with water, dried using Na<sub>2</sub>SO<sub>4</sub> and then concentrated to obtain the residue, which was purified using column chromatography (6% MeOH/dichloromethane) to obtain the compound **51** (133 mg). To a solution of **51** in a solvent mixture of MeOH:dichloromethane:chloroform (0.1:1:1), 3-chloroperoxybenzoic acid (138 mg, 0.8 mmol) was added and the solution was refluxed at 45-50 °C for 40 minutes. The solvent was then removed under vacuum and the residue was purified using column chromatography (10% MeOH/dichloromethane) to obtain the *N*-oxide derivative (31 mg). This was then dissolved in anhydrous dichloromethane, followed by the addition of benzoyl

isocyanate (16 mg, 0.11 mmol) and heated at 45 °C for 15 minutes. The solvent was then removed under vacuum and the residue was dissolved in anhydrous MeOH, followed by the addition of excess sodium methoxide and heated at 80 °C for 1 hour. The solvent was then removed under vacuum and the residue was purified using column chromatography (8% MeOH/dichloromethane) to obtain the intermediate *N*-Boc derivative. This was dissolved in 5 mL of trifluoroacetic acid and stirred for 30 minutes, followed by removal of the solvent by purging nitrogen and drying under vacuum to obtain the trifluoroacetate salt of the compound **52** (26 mg, 20%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.02 (d, *J* = 8.4 Hz, 1H), 7.82 (d, *J* = 8.4 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.49 – 7.32 (m, 4H), 7.15 (d, *J* = 7.6 Hz, 2H), 6.00 (s, 2H), 4.71 (s, 2H), 3.38 (q, *J* = 7.3 Hz, 2H), 1.45 (t, *J* = 7.3 Hz, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 149.52, 148.45, 136.86, 134.46, 134.03, 130.18, 129.15, 128.12, 125.29, 125.15, 124.97, 121.76, 118.34, 112.47, 48.94, 43.17, 42.36, 10.06. MS (ESI) calculated for C<sub>20</sub>H<sub>21</sub>N<sub>5</sub>, *m/z* 331.18, found 332.19 (*M* + H)<sup>+</sup>.

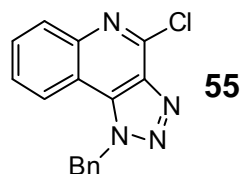
**Synthesis of Compound 54: 1-(4-Amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)-2-methylpropan-2-ol.**



To a solution of **20** (100 mg, 0.43 mmol) in anhydrous THF, were added triethylamine (66 mg, 0.65 mmol) and valeryl chloride (62 mg, 0.52 mmol). The reaction mixture was then stirred for 6-8 hours, followed by removal of the solvent under vacuum. The residue was dissolved in EtOAc, washed with water, brine and then dried over Na<sub>2</sub>SO<sub>4</sub> to obtain the intermediate amide compound. This was dissolved in MeOH, followed by the addition of calcium oxide and was heated in microwave at 110 °C for 1 hour. The solvent was then removed and the residue was purified using column chromatography (9% MeOH/dichloromethane) to obtain the compound **53** (58 mg). To a solution of **53** in a solvent mixture of MeOH:dichloromethane:chloroform (0.1:1:1), was added 3-

chloroperoxybenzoic acid (84 mg, 0.49 mmol) and the solution was refluxed at 45-50 °C for 40 minutes. The solvent was then removed and the residue was purified using column chromatography (20% MeOH/dichloromethane) to obtain the *N*-oxide derivative (55 mg). This was then dissolved in anhydrous dichloromethane, followed by the addition of benzoyl isocyanate (39 mg, 0.26 mmol) and heated at 45 °C for 15 minutes. The solvent was then removed under vacuum and the residue was dissolved in anhydrous MeOH, followed by the addition of excess sodium methoxide. The reaction mixture was then heated at 80 °C for an hour. The solvent was removed under vacuum and the residue was purified using column chromatography (11% MeOH/dichloromethane) to obtain the compound **54** (40 mg, 30%). <sup>1</sup>H NMR (400 MHz, MeOD) δ 8.26 (d, *J* = 8.3 Hz, 1H), 7.69 (d, *J* = 8.4 Hz, 1H), 7.47 (t, *J* = 7.7 Hz, 1H), 7.31 (t, *J* = 7.7 Hz, 1H), 4.63 (s, 2H), 3.20 – 3.02 (m, 2H), 1.98 – 1.80 (m, 2H), 1.62 – 1.45 (m, 2H), 1.27 (s, 6H), 1.04 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 156.33, 151.22, 143.80, 134.53, 126.75, 125.41, 125.12, 121.49, 120.93, 115.31, 71.16, 54.80, 29.72, 27.12, 26.52, 22.24, 12.86. MS (ESI) calculated for C<sub>18</sub>H<sub>24</sub>N<sub>4</sub>O, *m/z* 312.20, found 313.20 (*M* + H)<sup>+</sup>.

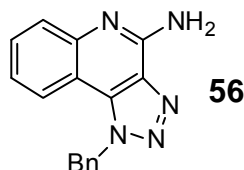
**Synthesis of Compound 55: 1-Benzyl-4-chloro-1*H*-[1,2,3]triazolo[4,5-*c*]quinoline.**



To a solution of **27** (60 mg, 0.21 mmol) in 1.5 mL of 1:1 acetic acid/water at 0 °C, was added 0.5N aqueous solution of sodium nitrite (0.56 mL, 0.28 mmol) and the reaction was stirred for 2 hours. The solvent was then removed under vacuum. The residue was dissolved in EtOAc, washed with water and dried over Na<sub>2</sub>SO<sub>4</sub>. The EtOAc fraction was then concentrated under vacuum to obtain the compound **55** (58 mg, 94%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.12 (dd, *J* = 8.4, 0.7 Hz, 1H), 7.96 (dd, *J* = 8.2, 0.9 Hz, 1H), 7.70 (ddd, *J* = 8.4, 7.2, 1.4 Hz, 1H), 7.52 (ddd, *J* = 8.3, 7.2, 1.2 Hz, 1H), 7.30 – 7.21 (m, 3H), 7.12 – 7.09 (m, 2H), 6.19 (s, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 142.94, 141.16, 136.74, 132.71, 131.61, 128.30, 127.80, 127.24, 126.61, 125.80, 124.24, 124.20, 120.01, 112.71,

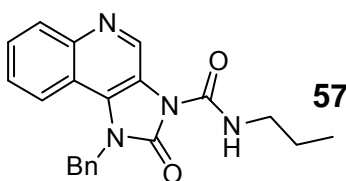
52.00. MS (ESI) calculated for  $C_{16}H_{11}ClN_4$ ,  $m/z$  294.07, found 317.06 ( $M + Na^+$ ) and 612.11 ( $M + M + Na^+$ ).

**Synthesis of Compound 56: 1-Benzyl-1*H*-[1,2,3]triazolo[4,5-*c*]quinolin-4-amine.**



Compound **55** (46 mg, 0.16 mmol) was dissolved in 0.1 mL of 2M ammonia solution in MeOH and the reaction was heated in microwave at 150 °C for 1 hour. The solvent was then removed under vacuum and the residue was purified using column chromatography (65% EtOAc/hexane) to obtain the compound **56** (35 mg, 82%).  $^1H$  NMR (500 MHz, TFA)  $\delta$  8.25 – 8.13 (m, 1H), 7.87 (d,  $J$  = 7.2 Hz, 1H), 7.79 (d,  $J$  = 8.0 Hz, 1H), 7.63 (t,  $J$  = 6.1 Hz, 1H), 7.39 (s, 3H), 7.24 (s, 2H), 6.38 (d,  $J$  = 23.6 Hz, 2H).  $^{13}C$  NMR (126 MHz, TFA)  $\delta$  134.33, 133.27, 131.52, 129.18, 129.06, 127.08, 125.83, 123.58, 118.48, 117.54, 115.29, 113.04, 110.78, 54.87. MS (ESI) calculated for  $C_{16}H_{11}N_5$ ,  $m/z$  275.12, found 276.12 ( $M + H$ ) $^+$ .

**Synthesis of Compound 57: 1-Benzyl-2-oxo-*N*-propyl-1*H*-imidazo[4,5-*c*]quinoline-3(2*H*)-carboxamide.**



To a solution of **41** (30 mg, 0.12 mmol) in anhydrous THF, were added triethylamine (15 mg, 0.14 mmol) and *n*-propyl isocyanate (33 mg, 0.39 mmol); the *n*-propylamine released as a consequence of cyclization en route to the cyclic urea was found to consume *n*-propyl isocyanate, necessitating the use of additional equivalents of the reagent. The reaction was heated in microwave at 100 °C for 1 hour. The solvent was then removed under vacuum and the residue was purified using column

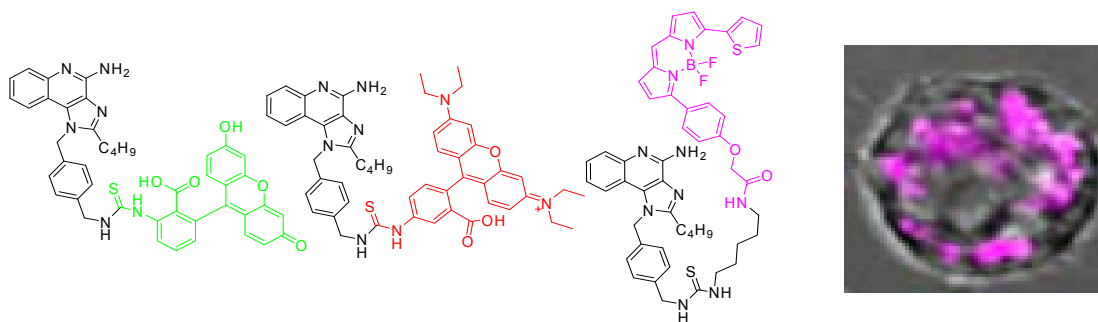
chromatography (6% MeOH/dichloromethane) to obtain the compound **57** (26 mg, 61%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  9.90 (s, 1H), 8.79 (s,  $J$  = 5.4 Hz, 1H), 8.15 (d,  $J$  = 8.5, 0.6 Hz, 1H), 7.91 (d,  $J$  = 8.6, 0.6 Hz, 1H), 7.60 (t,  $J$  = 8.4, 6.9, 1.3 Hz, 1H), 7.40 (t,  $J$  = 8.3, 6.9, 1.2 Hz, 1H), 7.37 – 7.32 (m, 2H), 7.31 – 7.28 (m, 1H), 7.26 (d,  $J$  = 6.7, 5.4 Hz, 2H), 5.60 (s, 2H), 3.49 – 3.45 (m,  $J$  = 7.1, 5.8 Hz, 2H), 1.78 – 1.68 (m, 2H), 1.04 (t,  $J$  = 7.4 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  151.08, 148.67, 143.44, 136.25, 132.69, 128.54, 127.08, 127.02, 125.87, 125.74, 124.61, 123.68, 118.43, 117.87, 112.78, 44.45, 39.68, 20.57, 9.22. MS (ESI) calculated for  $\text{C}_{21}\text{H}_{20}\text{N}_4\text{O}_2$ ,  $m/z$  360.16, found 361.17 ( $\text{M} + \text{H}$ ) $^+$ .

**TLR7/8 Reporter Gene assays (NF- $\kappa$ B induction):** The induction of NF- $\kappa$ B was quantified using HEK-Blue-7 cells and HEK-Blue-8 cells as previously described by us.<sup>69;137</sup> HEK293 cells were stably transfected with human TLR7 (or human TLR8), MD2, and secreted alkaline phosphatase (sAP), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF- $\kappa$ B/AP-1 promoters is inducible by the TLR7 (or TLR8) agonists, and extracellular sAP in the supernatant is proportional to NF- $\kappa$ B induction. HEK-Blue cells were incubated at a density of  $\sim 10^5$  cells/mL in a volume of 80  $\mu\text{L}$ /well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

**IFN- $\alpha$  induction in whole human blood:** Aliquots (3 mL) of heparin-anticoagulated blood obtained from healthy human donors after informed consent and stimulated for 12 h with graded concentrations of test compounds. The plasma was isolated by centrifugation, diluted 1:20, and IFN- $\alpha$  was assayed in triplicate using a high-sensitivity human IFN- $\alpha$ -specific ELISA kit (PBL Interferon Source, Piscataway, NJ).

## Chapter 5.

# SYNTHESES OF FLUORESCENT IMIDAZOQUINOLINE CONJUGATES AS PROBES OF TOLL-LIKE RECEPTOR 7



## 5.1. Introduction.

Our point of departure in the systematic evaluation of TLR agonists as vaccine adjuvants<sup>69;137;150</sup> focuses, as described in the preceding chapters, on identifying chemotypes which are strongly immunostimulatory, and yet devoid of dominant proinflammatory cytokine-inducing activities;<sup>69</sup> the TLR7-agonistic imidazoquinolines have thus far seemed ideal in meeting these requirements.<sup>69</sup> Continuing work on characterizing the immunostimulatory activities of TLR7 agonists show, as expected from its immunostimulatory profile (see Chapters 1-2, ref. 69), clear involvement of plasmacytoid dendritic cells;<sup>174</sup> however, we have also observed a set of accessory cell-independent direct responses in CD4<sup>+</sup> and CD8<sup>+</sup> T and CD3<sup>-</sup>CD56<sup>+</sup> natural killer (NK) lymphocytes (not discussed in this thesis). We were specifically desirous of examining the uptake, intracellular distribution, and trafficking of the imidazoquinoline in immunological synapses,<sup>175;176</sup> and it became necessary to develop probes of TLR7 that are fluorescently labeled.

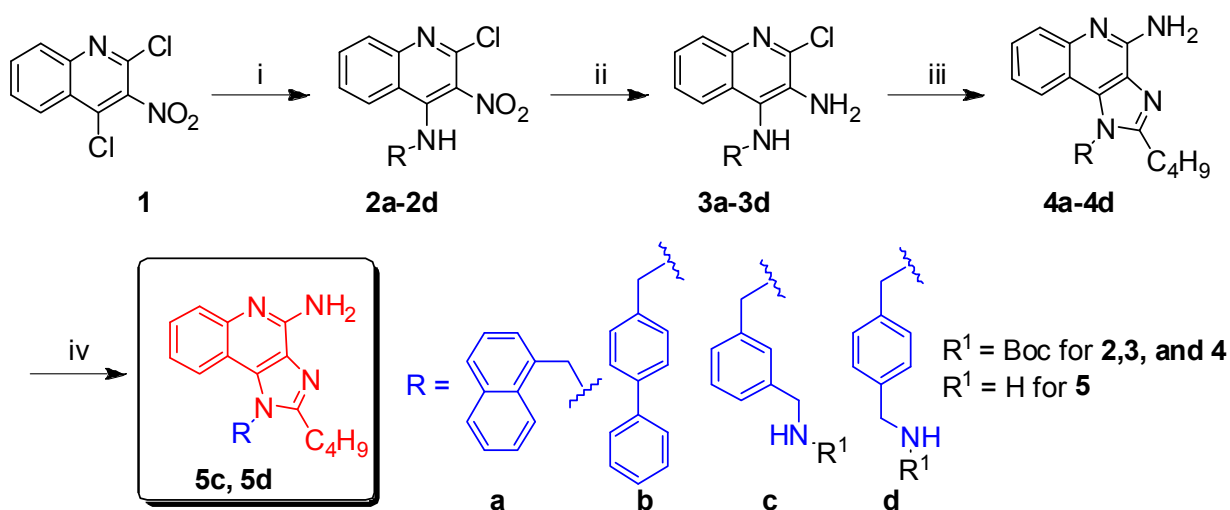
Our earlier SAR study on the TLR7-agonistic activities (Chapter 4) had exhaustively explored C2 and C4 substituents on the imidazoquinoline scaffold, but proved unsuccessful in identifying potential positions that would tolerate the introduction of bulky aryl groups without compromising activity. Our attention subsequently turned to exploring the effect of varying substituents at *N*<sup>1</sup>, while holding the C2-*n*-butyl and C4-NH<sub>2</sub> groups constant since these have been shown to correspond to maximal activity.<sup>132</sup> The synthesis of a TLR7-active *N*<sup>1</sup>-(4-aminomethyl)benzyl substituted imidazoquinoline served as a convenient precursor for the covalent attachment of fluorophores without significant loss of activity. Fluorescence microscopy experiments show that the fluorescent analogues are internalized and distributed in the endosomal compartment. Flow cytometry experiments using whole human blood show differential partitioning into B, T, and natural killer (NK) lymphocytic subsets, which correlate with the degree of activation in these subsets. These fluorescently-labeled imidazoquinolines will likely be useful in examining in detail the trafficking of TLR7 in immunological synapses.



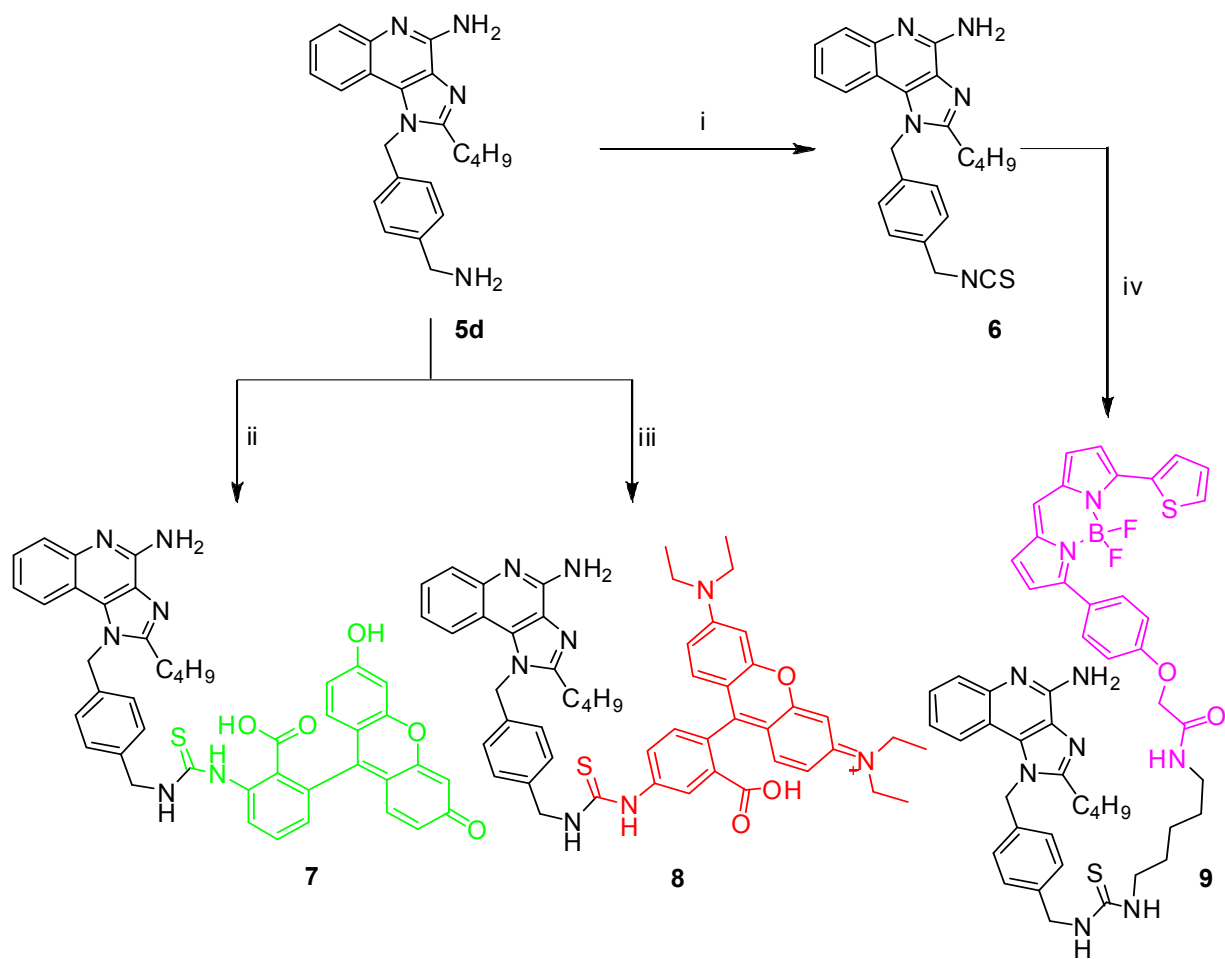
## 5.2. Results and Discussion.

The  $N^1$ -naphthylenemethyl-substituted compound **4a** was inactive, and the  $N^1$ -biphenyl-4-methyl compound **4b** was weakly active ( $EC_{50}$ : 396 nM); the  $N^1$ -(4-aminomethyl)benzyl substituted analogue **5d** was considerably more active ( $EC_{50}$ : 20 nM; Scheme 1) than its  $N^1$ -(3-aminomethyl)benzyl regioisomer **5c** ( $EC_{50}$ : 110 nM). The free primary amine on the  $N^1$  substituent of **5d** was covalently coupled directly to commercially-available fluorescein isothiocyanate and rhodamine B isothiocyanate (Scheme 2). Conversely, the amine on **5d** was converted first to the isothiocyanate **6**, allowing the subsequent coupling of amine-bearing fluorophores, such as the bora-diazaindacene dye, BODIPY-TR-cadaverine (Scheme 2, overleaf).

**Scheme 1.** Syntheses of  $N^1$ -substituted imidazoquinolines.



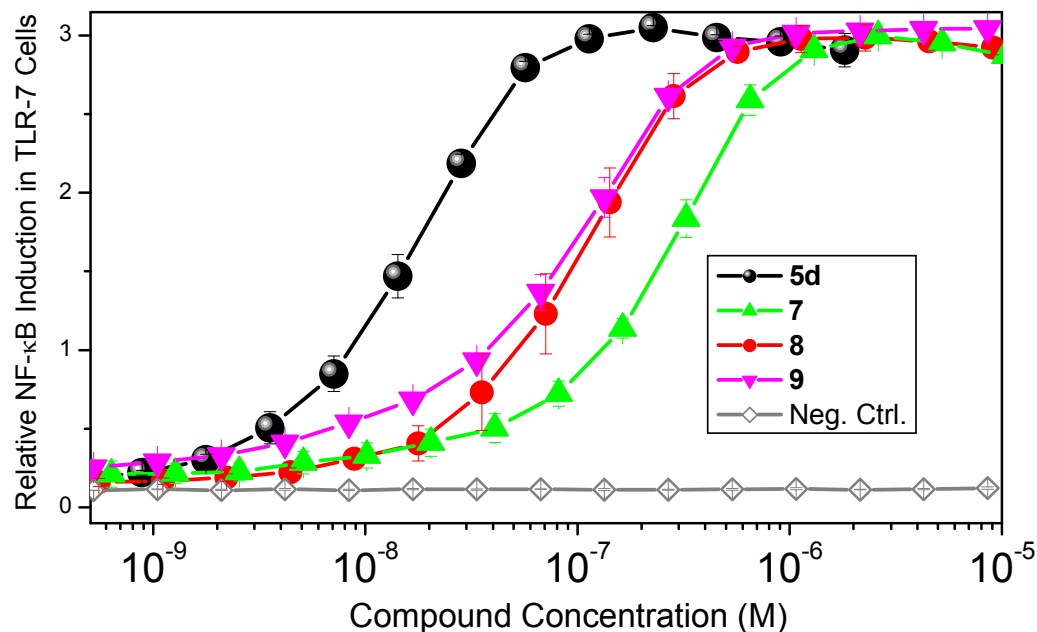
**Scheme 2.** Syntheses of fluorescent analogues of **5d**.



Reagents: *i*. CS<sub>2</sub>, Et<sub>3</sub>N, DMAP, (Boc)<sub>2</sub>O, CH<sub>2</sub>Cl<sub>2</sub>; *ii*. Fluorescein isothiocyanate, Et<sub>3</sub>N, MeOH; *iii*. Rhodamine B isothiocyanate, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>; *iv*. BODIPY<sup>®</sup> TR cadaverine, pyridine.

All three fluorescent conjugates retain TLR7-agonistic activity, although their potencies are slightly attenuated relative to the parent compound, **5d** (Fig. 1).

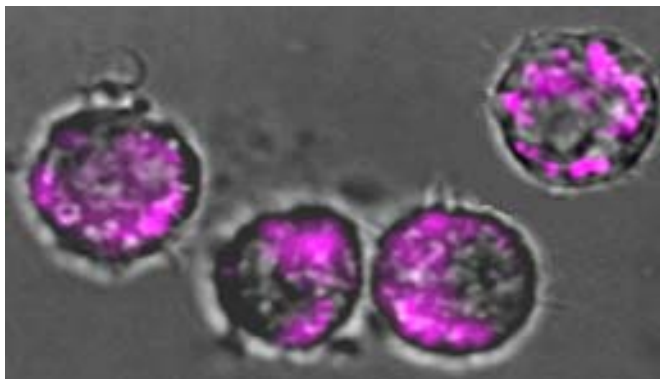
**Fig. 1.** Activities of **5d**, **7**, **8**, and **9** in reporter gene assays using human TLR7.



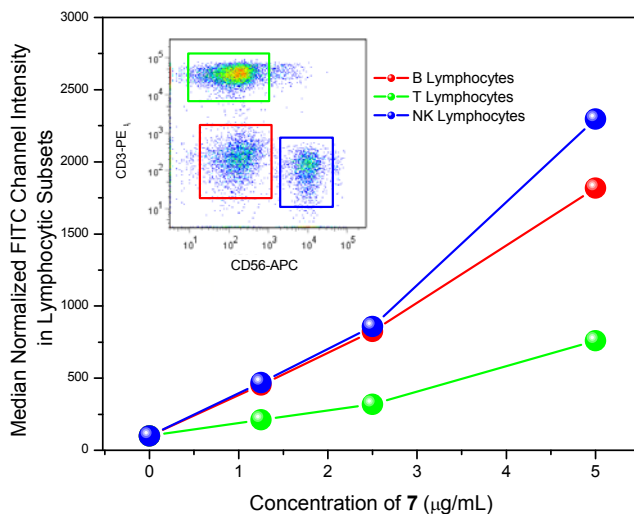
Incubation of murine macrophage J774.A1 cells with **8** and **9**, followed by intravital epi- and confocal fluorescence microscopy showed prominent perinuclear localization, which is consistent with the expected endosomal distribution of TLR7.<sup>177</sup> Shown in **Fig. 2** is a representative epifluorescence micrograph of J774 cells treated with **9** at 100 nM concentration.

Our earlier immunoprofiling of the TLR7-agonistic imidazoquinolines had shown a very prominent activation of B- and NK-cells, but minimal activation of T cells,<sup>69</sup> and we asked if a possible reason could be differential uptake of the TLR7 agonist in lymphocytic subsets. Flow cytometric analysis of the FITC-labeled **7** in experiments employing whole human blood indeed show a prominent uptake of **7** in CD3<sup>+</sup>CD56<sup>+</sup> NK and CD3<sup>+</sup>CD56<sup>-</sup> B lymphocytes as compared to CD3<sup>+</sup>CD56<sup>-</sup> T lymphocytes (**Fig. 3**).

**Fig. 2.** Murine J774 cells treated with 100 nM of **9**. An overlay of phase-contrast and epifluorescence images is depicted. An excitation filter at 562nm and a long-pass emission filter (601-800) were used.



**Fig. 3.** Uptake of **7** in lymphocytic subsets as examined by flow cytometry. Whole human blood was incubated with graded concentrations of **7** for 30 min, lymphocytes stained with cell surface markers (anti-CD3-phycoerythrin [PE], and anti-CD56-PE-allophycocyanin). Erythrocytes were lysed, and  $10^5$  total events were acquired per sample.



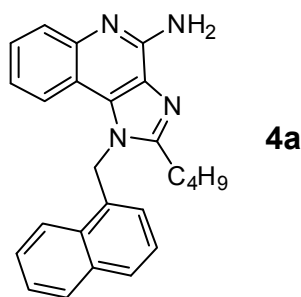
### 5.3. Conclusions.

The syntheses of fluorescent imidazoquinoline analogues that retain TLR7-agonistic activity are expected to be useful probes in examining the anatomical basis of their potential immunostimulatory and adjuvant properties. **5d** has also served as a convenient precursor for the synthesis of isothiocyanate and maleimide derivatives, enabling its direct conjugation to protein and polysaccharide antigens (Chapter 7).

## 5.4. Experimental.

All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instruments unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm x 150 mm, 5  $\mu$ m analytical reverse phase C<sub>18</sub> column with H<sub>2</sub>O-isopropanol or H<sub>2</sub>O-CH<sub>3</sub>CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion acquisition mode. All the compounds synthesized were obtained as solids.

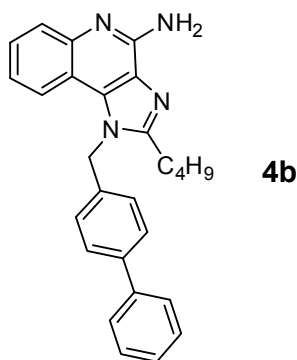
**Synthesis of Compound 4a: 2-Butyl-1-(naphthalen-1-ylmethyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



To a solution of **1** (100 mg, 0.41 mmol) in 5 mL of anhydrous dichloromethane, were added triethylamine (54 mg, 0.53 mmol) and naphthalen-1-ylmethanamine (71 mg, 0.45 mmol). The reaction mixture was refluxed at 45 °C for 30 minutes. The solvent was then evaporated under vacuum and product was isolated using column chromatography to obtain the intermediate compound **2a**. To a solution of **2a** in 10 mL of EtOAc, were added a catalytic amount of Pt/C and Na<sub>2</sub>SO<sub>4</sub>. The reaction mixture was subjected to hydrogenation at 55 psi hydrogen pressure for 4 hours. The reaction mixture was then filtered through celite and the filtrate was evaporated under

vacuum to obtain the compound **3a** (90 mg). To a solution of **3a** (90 mg, 0.27 mmol) in anhydrous THF, were added triethylamine (41 mg, 0.41 mmol) and valeryl chloride (39 mg, 0.32 mmol). The reaction mixture was stirred at room temperature for 6 hours. The solvent was then removed under vacuum, and the residue was dissolved in ethyl acetate and washed with water. The ethyl acetate fraction was then dried using Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum to obtain the intermediate amide compound, which was then dissolved in 2 mL of 2M solution of ammonia in MeOH. The sealed reaction vessel was heated 150 °C for 24 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (7% MeOH/dichloromethane) to obtain the compound **4a** (62 mg; 40%). <sup>1</sup>H NMR (500 MHz, DMSO) δ 8.40 (d, *J* = 8.3 Hz, 1H), 8.05 (d, *J* = 8.1 Hz, 1H), 7.86 (d, *J* = 8.2 Hz, 1H), 7.77 (t, *J* = 7.2 Hz, 1H), 7.69 (t, *J* = 7.5 Hz, 1H), 7.62 (d, *J* = 8.2 Hz, 1H), 7.47 (d, *J* = 8.2 Hz, 1H), 7.33 (t, *J* = 7.5 Hz, 1H), 7.27 (t, *J* = 7.7 Hz, 1H), 7.08 (s, 2H), 6.90 (t, *J* = 7.5 Hz, 1H), 6.39 (d, *J* = 7.1 Hz, 1H), 6.35 (s, 2H), 2.92 (t, *J* = 7.6 Hz, 2H), 1.69 (dt, *J* = 15.2, 7.6 Hz, 2H), 1.32 (dt, *J* = 14.6, 7.4 Hz, 2H), 0.80 (t, *J* = 7.3 Hz, 3H). <sup>13</sup>C NMR (126 MHz, DMSO) δ 154.39, 151.16, 133.62, 133.25, 131.77, 129.64, 128.74, 127.88, 126.91, 126.80, 126.48, 126.15, 125.58, 123.07, 121.57, 121.42, 119.87, 114.11, 46.60, 29.45, 26.08, 21.70, 13.61. MS (ESI) calculated for C<sub>25</sub>H<sub>24</sub>N<sub>4</sub>, *m/z* 380.20, found 381.21 (*M* + H)<sup>+</sup>.

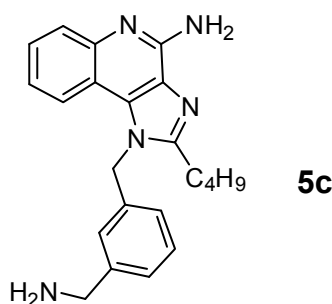
**Compound 4b was synthesized similarly as described for compound 4a.**



**4b: 1-(Biphenyl-4-ylmethyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine.** <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.88 (d, *J* = 7.9 Hz, 1H), 7.78 – 7.73 (m, 1H), 7.60 – 7.56 (m, 2H), 7.55 – 7.52 (m, 2H), 7.51 – 7.47 (m, 1H), 7.45 – 7.40 (m, 2H), 7.35 (ddt, *J* = 8.5, 6.5, 1.4 Hz, 1H), 7.30 – 7.25 (m, 1H), 7.10 (d, *J* = 8.3 Hz, 2H), 5.81 (s, 2H), 2.94 – 2.91 (m, 2H), 1.84 (dt, *J* = 15.4, 7.6 Hz, 2H), 1.51 – 1.41 (m,

2H), 0.95 (t,  $J = 7.4$  Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  156.27, 149.52, 141.25, 139.59, 135.17, 132.65, 129.02, 128.64, 127.93, 127.52, 126.72, 125.52, 124.77, 124.58, 120.31, 120.23, 112.54, 48.72, 29.17, 26.80, 22.15, 13.47. MS (ESI) calculated for  $\text{C}_{27}\text{H}_{26}\text{N}_4$ ,  $m/z$  406.22, found 407.22 ( $\text{M} + \text{H}$ ) $^+$ .

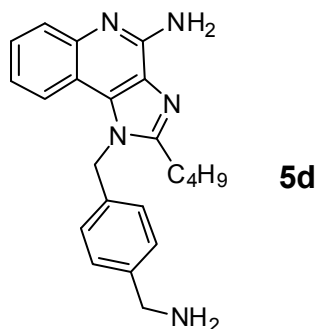
**Synthesis of Compound 5c: 1-(3-(Aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine.**



To a solution of **1** (200 mg, 0.83 mmol) in 5 mL of anhydrous dichloromethane, were added triethylamine (92 mg, 0.91 mmol) and *tert*-butyl 3-(aminomethyl)benzylcarbamate (215 mg, 1.06 mmol) dissolved in 2 mL of anhydrous MeOH. The reaction mixture was refluxed at 45  $^{\circ}\text{C}$  for 30 minutes. The solvent was then evaporated under vacuum and product was isolated using column chromatography to obtain the intermediate compound **2c**. To a solution of **2c** in 10 mL of EtOAc, were added a catalytic amount of Pt/C and  $\text{Na}_2\text{SO}_4$ . The reaction mixture was subjected to hydrogenation at 55 psi hydrogen pressure for 4 hours. The reaction mixture was then filtered through celite and the filtrate was evaporated under vacuum to obtain the compound **3c** (202 mg). To a solution of **3c** (202 mg, 0.49 mmol) in anhydrous THF, were added triethylamine (64 mg, 0.64 mmol) and valeryl chloride (73 mg, 0.54 mmol). The reaction mixture was stirred at room temperature for 6 hours. The solvent was then removed under vacuum, and the residue was dissolved in ethyl acetate and washed with water. The ethyl acetate fraction was then dried using  $\text{Na}_2\text{SO}_4$  and evaporated under vacuum to obtain the intermediate amide compound, which was then dissolved in 2 mL of 2M solution of ammonia in MeOH. The sealed reaction vessel was heated 150  $^{\circ}\text{C}$  for 24 hours. The solvent was then removed under vacuum and the residue was purified using

column chromatography (9% MeOH/dichloromethane) to obtain the compound **4c** (44 mg; 12%). This was then dissolved in 10 mL of HCl/dioxane solution and stirred for 12 hours. The solvent was then removed to obtain the compound **5c** (52 mg, 15%).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  7.85 (s, 1H), 7.67 (d,  $J$  = 7.0 Hz, 1H), 7.52 (s, 1H), 7.39 – 7.18 (m, 4H), 7.02 (s, 1H), 5.92 (s, 2H), 4.01 (s, 2H), 2.94 (s, 2H), 1.80 (s, 2H), 1.41 (d,  $J$  = 4.4 Hz, 2H), 0.88 (t,  $J$  = 6.1 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  159.02, 150.28, 137.51, 135.84, 135.26, 131.34, 131.06, 129.95, 127.66, 127.46, 126.67, 125.73, 123.01, 119.66, 114.08, 50.24, 44.18, 30.32, 27.98, 23.45, 14.25. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{25}\text{N}_5$ ,  $m/z$  359.21, found 360.22 ( $\text{M} + \text{H}$ ) $^+$ .

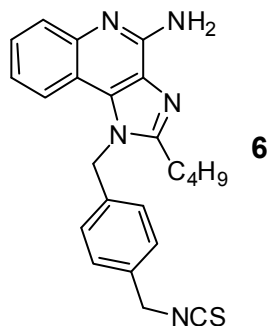
**Compound 5d was synthesized similarly as described for compound 5c.**



**5d: 1-(4-(Aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine.**  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  7.85 (d,  $J$  = 8.2 Hz, 1H), 7.69 (d,  $J$  = 8.3 Hz, 1H), 7.54 (t,  $J$  = 7.7 Hz, 1H), 7.40 (d,  $J$  = 7.7 Hz, 2H), 7.26 (t,  $J$  = 7.6 Hz, 1H), 7.10 (d,  $J$  = 7.8 Hz, 2H), 5.93 (s, 2H), 4.01 (s, 2H), 2.94 (t,  $J$  = 7.6 Hz, 2H), 1.83 – 1.71 (m, 2H), 1.43 – 1.32 (m, 2H), 0.86 (t,  $J$  = 7.3 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  159.02, 150.27, 137.51, 137.47, 135.33, 134.59, 131.17, 131.11, 127.54, 126.51, 125.53, 122.95, 119.66, 114.03, 49.93, 43.81, 30.31, 27.78, 23.35, 14.12. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{25}\text{N}_5$ ,  $m/z$  359.21, found 360.22 ( $\text{M} + \text{H}$ ) $^+$ .

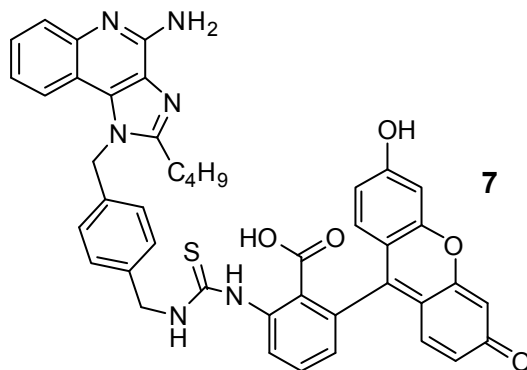


**Synthesis of Compound 6: 2-Butyl-1-(4-(isothiocyanatomethyl)benzyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



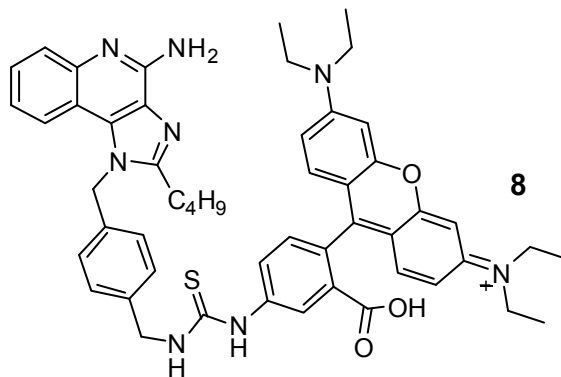
To a solution of **5d** (150 mg, 0.35 mmol) in anhydrous dichloromethane, were added carbon disulfide (266 mg, 3.5 mmol) and triethylamine (106 mg, 1.05 mmol). The reaction mixture was stirred for an hour and then was cooled to 0 °C. Di-*tert*-butyl dicarbonate (76 mg, 0.35 mmol) and a catalytic amount of DMAP were added to the reaction mixture. The reaction mixture was stirred for 18 hours and then the solvent was removed under vacuum. The residue was purified using column chromatography (7% MeOH/ dichloromethane) to obtain the compound **6** (105 mg, 75%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.87 – 7.83 (m, 1H), 7.68 (dd, *J* = 8.3, 0.8 Hz, 1H), 7.51 – 7.45 (m, 1H), 7.32 (d, *J* = 8.2 Hz, 2H), 7.23 – 7.17 (m, 1H), 7.10 (d, *J* = 8.2 Hz, 2H), 6.52 (s, 2H), 5.78 (s, 2H), 4.71 (s, 2H), 2.94 – 2.86 (m, 2H), 1.82 (dt, *J* = 15.5, 7.6 Hz, 2H), 1.52 – 1.41 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 155.19, 150.27, 135.10, 134.65, 134.57, 128.20, 127.95, 126.10, 125.92, 124.08, 123.59, 119.94, 113.99, 48.74, 48.21, 29.71, 27.12, 22.47, 13.76. MS (ESI) calculated for C<sub>23</sub>H<sub>23</sub>N<sub>5</sub>S, *m/z* 401.17, found 402.18 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 7: 2-(3-(4-((4-Amino-2-butyl-1*H*-imidazo [4,5-*c*]quinolin-1-yl)methyl)benzyl)thioureido)-6-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid.**



To a solution of fluorescein isothiocyanate (17 mg, 0.043 mmol) in anhydrous MeOH, were added triethylamine (13 mg, 0.13 mmol) and **5d** (20mg, 0.043 mmol). The reaction mixture was then heated at 45 °C for 18 hours and then the solvent was removed under vacuum. The residue was then purified using column chromatography (22% MeOH/dichloromethane) to obtain the compound **7** (3 mg, 10%). <sup>1</sup>H NMR (500 MHz, DMSO)  $\delta$  10.13 (s, 3H), 8.44 (s, 1H), 8.21 (s, 1H), 7.88 – 7.67 (m, 3H), 7.62 – 7.53 (m, 1H), 7.33 (t,  $J$  = 8.1 Hz, 2H), 7.26 – 7.13 (m, 2H), 7.07 – 6.94 (m, 3H), 6.67 (d,  $J$  = 2.1 Hz, 2H), 6.57 (tt,  $J$  = 5.4, 4.0 Hz, 5H), 5.87 (s, 2H), 4.74 (s, 2H), 2.97 – 2.84 (m, 2H), 1.77 – 1.67 (m, 2H), 1.45 – 1.33 (m, 2H), 0.91 – 0.85 (m, 3H). MS (ESI) calculated for C<sub>43</sub>H<sub>36</sub>N<sub>6</sub>O<sub>5</sub>S,  $m/z$  748.25, found 749.26 (M + H)<sup>+</sup>.

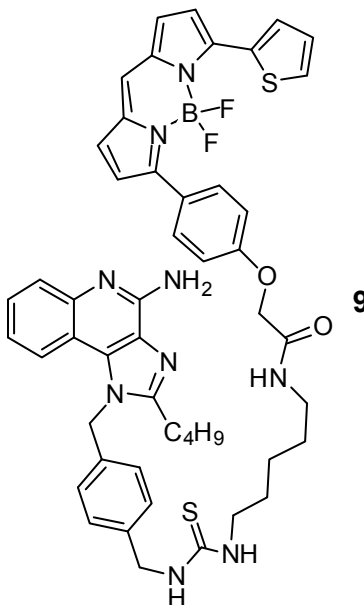
**Synthesis of Compound 8:** *N*-(9-(4-(3-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzyl)thioureido)-2-carboxyphenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium.



To a solution of rhodamine B isothiocyanate (50 mg, 0.12 mmol) in anhydrous dichloromethane, were added triethylamine (47 mg, 0.47 mmol) and **5d** (64 mg, 0.12 mmol). The reaction mixture was then

stirred for 14 hours and then the solvent was removed under vacuum. The residue was then purified using column chromatography (50% MeOH/dichloromethane) to obtain the compound **8** (16 mg, 16%).  $^1\text{H}$  NMR (500 MHz, DMSO)  $\delta$  10.06 (s, 1H), 8.49 (s, 2H), 7.84 (d,  $J$  = 8.4 Hz, 1H), 7.81 – 7.73 (m, 2H), 7.56 (dd,  $J$  = 8.4, 1.0 Hz, 2H), 7.33 – 7.29 (m, 1H), 7.24 (d,  $J$  = 8.1 Hz, 2H), 7.03 – 6.95 (m, 3H), 6.51 (dd,  $J$  = 12.7, 8.4 Hz, 4H), 6.46 – 6.41 (m, 4H), 5.82 (s, 2H), 4.63 (s, 2H), 3.36 (dd,  $J$  = 11.9, 4.8 Hz, 8H), 2.92 – 2.83 (m, 2H), 1.70 (dt,  $J$  = 15.3, 7.6 Hz, 2H), 1.36 (dq,  $J$  = 14.7, 7.4 Hz, 2H), 1.10 (t,  $J$  = 7.0 Hz, 12H), 0.85 (t,  $J$  = 7.4 Hz, 3H). MS (ESI) calculated for  $\text{C}_{51}\text{H}_{55}\text{N}_8\text{O}_3\text{S}^+$ ,  $m/z$  859.41, found 859.41 ( $\text{M}$ ) $^+$ .

### Synthesis of Compound 9: BODIPY<sup>®</sup>-TR cadaverine conjugated to Compound 6.



To a solution of BODIPY<sup>®</sup> TR cadaverine [5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)pentylamine] hydrochloride (Invitrogen, Inc., 10 mg, 0.02 mmol) in anhydrous pyridine, was added **6** (11 mg, 0.03 mmol). The reaction mixture was then heated at 45 °C for 18 hours and the solvent was then removed under vacuum. The residue was purified using column chromatography (8% MeOH/dichloromethane) to obtain the compound **9** (2.34 mg, 15%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  7.99 – 7.93 (m, 3H), 7.82 (d,  $J$  = 8.3 Hz, 1H), 7.66 (d,  $J$  = 8.4 Hz, 1H), 7.56 (t,  $J$  = 6.7 Hz, 2H), 7.42 (s, 1H), 7.26 (t,  $J$  = 7.3 Hz, 3H), 7.17 (dd,  $J$  = 8.5, 4.3 Hz, 2H), 7.06 (dd,

$J = 8.9, 2.6 \text{ Hz, 3H}$ ),  $6.95 \text{ (d, } J = 8.0 \text{ Hz, 2H)}$ ,  $6.83 \text{ (d, } J = 4.3 \text{ Hz, 1H)}$ ,  $6.74 \text{ (d, } J = 4.1 \text{ Hz, 1H)}$ ,  $5.73 \text{ (s, 2H)}$ ,  $4.57 \text{ (s, 2H)}$ ,  $3.22 \text{ (ddd, } J = 25.7, 16.1, 9.0 \text{ Hz, 4H)}$ ,  $2.88 - 2.83 \text{ (m, 2H)}$ ,  $1.76 \text{ (dt, } J = 15.3, 7.6 \text{ Hz, 3H)}$ ,  $1.51 \text{ (dt, } J = 18.8, 9.6 \text{ Hz, 4H)}$ ,  $1.39 \text{ (dd, } J = 15.1, 7.5 \text{ Hz, 2H)}$ ,  $1.36 - 1.24 \text{ (m, 3H)}$ ,  $0.90 \text{ (t, } J = 7.4 \text{ Hz, 3H)}$ . MS (ESI) calculated for  $\text{C}_{49}\text{H}_{50}\text{BF}_2\text{N}_9\text{O}_2\text{S}_2$ ,  $m/z$  909.36, found 910.37 ( $\text{M} + \text{H}$ )<sup>+</sup>.

**NF- $\kappa$ B induction in human TLR7-expressing reporter gene assays:** The induction of NF- $\kappa$ B was quantified using HEK-Blue-7 cells as previously described by us.<sup>137;178</sup> HEK293 cells were stably transfected with human TLR7, MD2, and secreted alkaline phosphatase (sAP), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF- $\kappa$ B/AP-1 promoters is inducible by the TLR7 agonists, and extracellular sAP in the supernatant is proportional to NF- $\kappa$ B induction. HEK-Blue cells were incubated at a density of  $\sim 10^5$  cells/mL in a volume of 80  $\mu\text{L}$ /well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

**Fluorescence microscopy:** Murine macrophage J774.A1 cells were grown to confluency in optical-grade flat-bottomed 96 well plates as described earlier.<sup>179;180</sup> The cells were then exposed to graded concentrations of the fluorescently labeled compounds for 4h at 37 °C. Intravital epifluorescence and phase contrast images were obtained directly from the plated cells using an inverted Olympus IX-71 microscope equipped with long working-distance air objectives and temperature-controlled stage, using appropriate filter sets for the various fluorescent analogues. Images were processed on Image-J software.

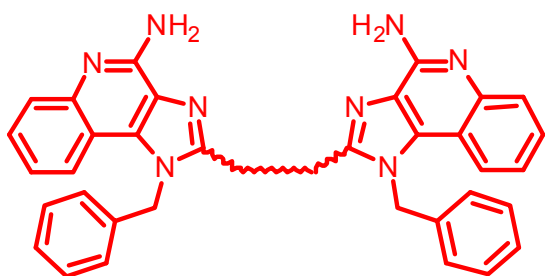
**Flow-cytometric immunostimulation experiments:** Detailed methods have been published by us.<sup>1</sup> Heparin-anticoagulated whole blood samples were obtained by venipuncture from healthy human volunteers with informed consent and as per guidelines approved by the University of Kansas

Human Subjects Experimentation Committee. Two mL aliquots of whole human blood samples were stimulated with graded concentrations of **7** in a 6-well polystyrene plate and incubated at 37 °C in a rotary (100 rpm) incubator for 30 min. Negative (endotoxin free water) controls were included in each experiment. Following incubation, 200 µL aliquots of anticoagulated whole blood were stained with 20 µL of fluorochrome-conjugated antibodies (anti-CD3-PE, and anti-CD56-APC) at 37 °C in the dark for 30 min. Following staining, erythrocytes were lysed and leukocytes fixed in one step by mixing 200 µL of the samples in 4 mL pre-warmed Whole Blood Lyse/Fix Buffer (Becton-Dickinson Biosciences, San Jose, CA). After washing the cells twice at 200g for 8 minutes in saline, the cells were transferred to a 96-well plate. Flow cytometry was performed using a BD FACSAArray instrument in the tri-color mode. The primary gate for the lymphocytic population was obtained on FSC and SSC channels (100,000 gated events). Secondary gating included natural killer lymphocytes (NK cells: CD3<sup>-</sup>CD56<sup>+</sup>), nominal B lymphocytes (CD3<sup>-</sup>CD56<sup>-</sup>), and nominal T lymphocytes (CD3<sup>+</sup>CD56<sup>-</sup>). Post-acquisition analyses were performed using FlowJo v 7.0 software (Treestar, Ashland, OR). Compensation for spillover was computed for each experiment on singly-stained samples.

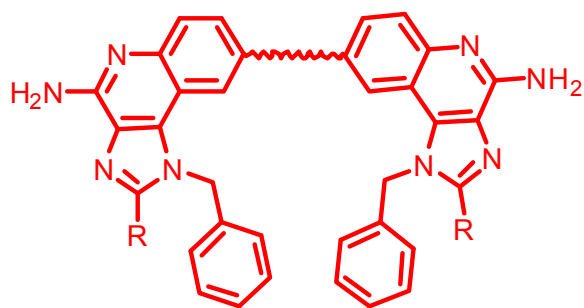
## Chapter 6.

# TLR3-, TLR7-, AND TLR8-MODULATORY ACTIVITIES OF IMIDAZOQUINOLINE DIMERS

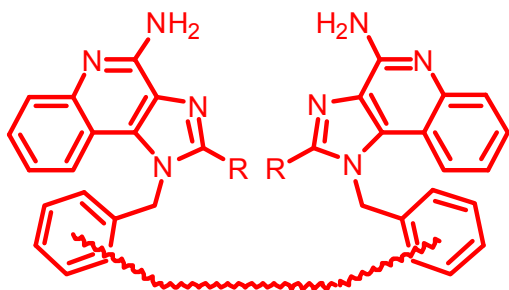
C2-Linked



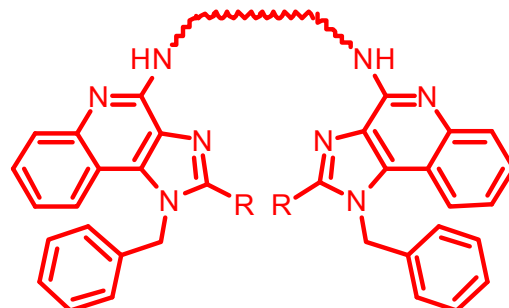
C8-Linked



*N*<sup>1</sup>-Benzyl Linked



C4-Amino Linked



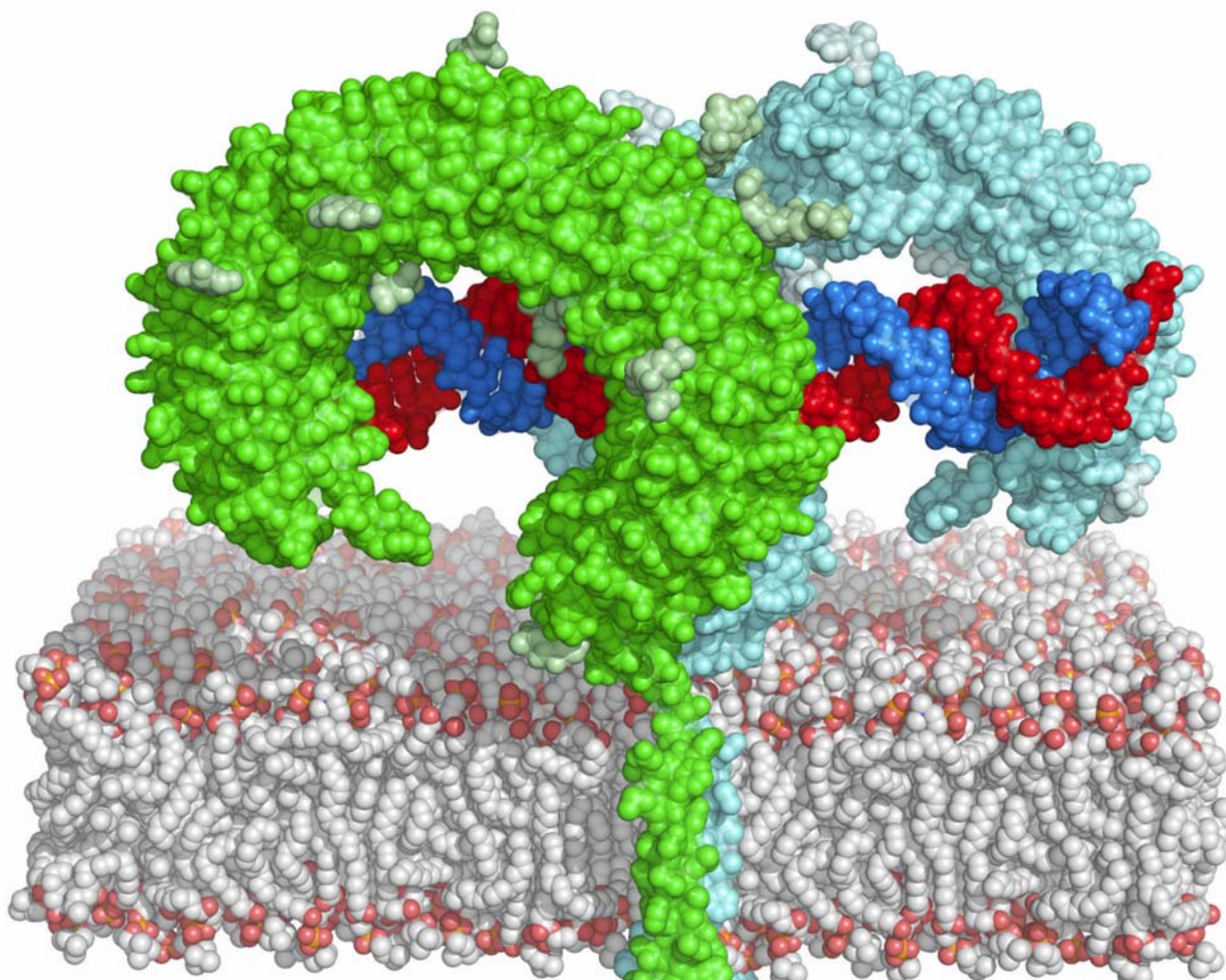
## 6.1. Introduction.

Unlike TLR7 and TLR8 which recognize single-stranded viral RNA (ssRNA), Toll-like receptor 3 (TLR3), also present in the endosomal compartment,<sup>181</sup> recognizes double-stranded RNA (dsRNA), a replication intermediate of many viruses, and triggers inflammatory responses characterized by the elaboration of type I interferon (IFN- $\alpha/\beta$ ) by virus-infected cells via activation of downstream NF- $\kappa$ B and IFN- $\beta$  promoters.<sup>182-187</sup> The secretion of IFN is a pivotal event in the induction of cellular antiviral immune responses.

A synthetic analog of naturally occurring dsRNA, polyriboinosinic:polyribocytidylic acid, poly(I:C), is often used experimentally to probe TLR3 responses.<sup>188</sup> The IFN-inducing properties of poly I:C has long been recognized, albeit empirically, predating the discovery of the involvement of TLR3 in its specific recognition by several decades. The safety profile of Ampligen®, also known as polyI:polyC12U [5'-Inosinic acid, homopolymer, complexed with 5'-cytidylic acid polymer with 5'-uridylic acid (1:1)] has been established in the context of HIV therapy, and has been found to be generally well-tolerated.<sup>189</sup> Poly(I:C(12)U) was found to be effective in inducing optimal phenotypic (elevated levels of MHC-Class I/Class II, CD83, CCR7, CD86 and CD40 molecules) and functional maturation of human DC *in vitro*, and capable of promoting the production of the Th1-type cytokine IL-12.<sup>190</sup> The production of Type I IFN is thought to be crucial in enhancing the primary antibody response to soluble proteins, and in the stimulation of the production of all subclasses of IgG, and consequent induction of long-lived antibody production and immunological memory.<sup>191</sup> Poly(I:C) also induced functional CD8<sup>+</sup> T-cell responses against OVA peptides in murine models; antigen-specific CD8<sup>+</sup> responses were found not only to be strongly IFN-I-dependent,<sup>192</sup> but also on the cytokine milieu provided by activated NK cells.<sup>193</sup> That poly I:C has strong adjuvant properties has been validated in multiple immunization models including the induction of mucosal (IgA-mediated) immunity.<sup>194-199</sup> TLR3, therefore, is an attractive target for adjuvant design and development.

The crystal structure of a complex between the ectodomains (ligand-binding region) of murine TLR3 and dsRNA has been determined at 3.4 Å resolution.<sup>200</sup> The TLR3 ectodomains bind dsRNA at two sites located at opposite ends of the TLR3 “horseshoe”, and an intermolecular contact between the two TLR3-ECD C-terminal domains coordinates and stabilizes the dimer (**Fig. 1**).

*Fig 1. dsRNA:TLR3 signaling complex. Murine TLR3 ectodomains (green and cyan) form a dimer on the dsRNA (blue and red). The N glycans are shown (light green and light blue). The proximity of the two C-termini permits association of the trans-membrane helices and the dimerization of the cytoplasmic domains (not shown). From Ref. 200.*

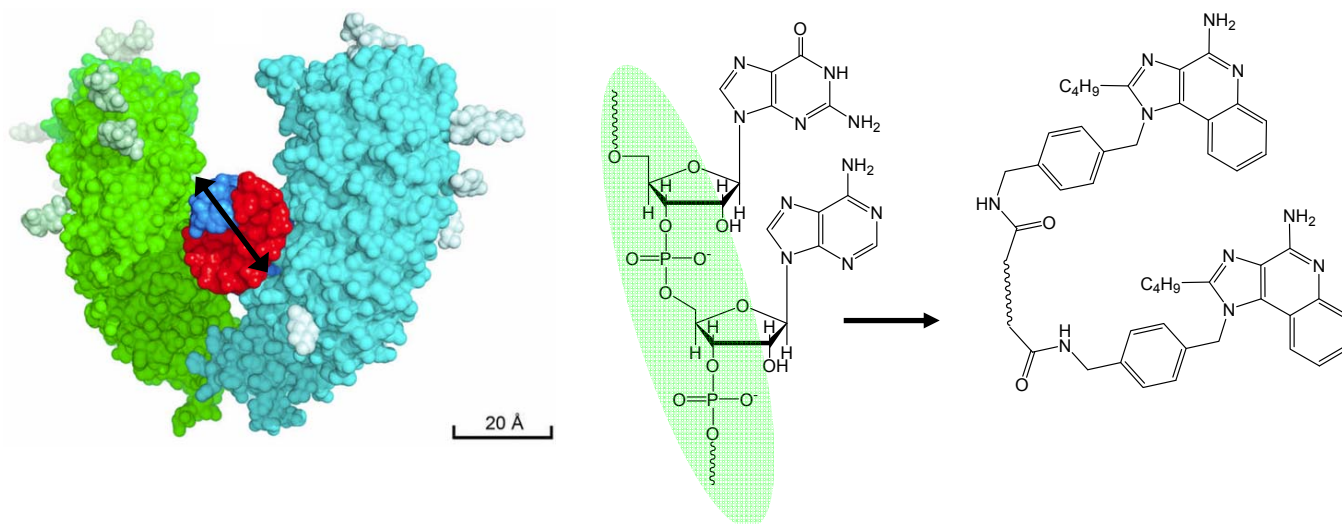




No small molecule ligands for TLR3 have been described to date. An inspection of the complex showed that the ribose-phosphate backbone but not the individual bases (which are base-paired, and, therefore inaccessible) is the major determinants of binding at the C-terminal binding site (**Fig. 2**).<sup>200</sup> However, the structural bases for discrimination of dsRNA and dsDNA by TLR3 and TLR9 remain unclear, and it is possible that additional recognition of the nucleobases may be contributory.

We were interested in constructing dimeric imidazoquinoline constructs with varying configurations purely as an intellectual exercise to examine whether any of such dimeric molecules would modulate (agonize or antagonize) TLR3 activities. This chapter describes the syntheses and characterization of dimeric imidazoquinolines on human TLR3, TLR7, and TLR8, as well as relevant secondary screens in human blood.

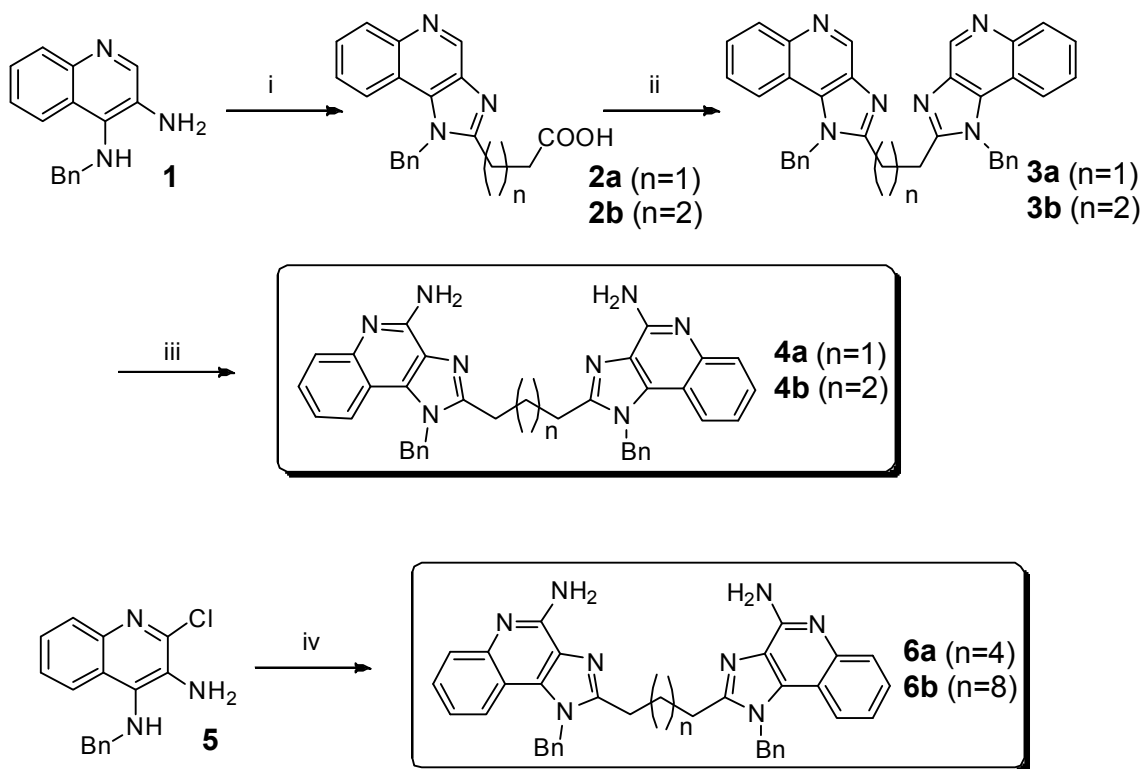
**Fig. 2.** Left: An orthogonal view of the dsRNA-TLR3 ectodomain complex showing interactions at the C-terminal binding site with the backbone of the dsRNA. From Ref. 200. Arrow shows possible recognition elements of a dimeric molecule of about 20Å in length. Center: proposed recognition motif of the RNA scaffold by one unit of TLR3. Right: proposed dimer of imidazoquinoline (one possible dimeric configuration shown).



## 6.2. Results and Discussion.

The first series of dimeric imidazoquinolines were linked at the C2 position and their syntheses necessitated two different routes. Whereas the hexamethylene- and decamethylene-bridged compounds **6a** and **6b** could be conveniently obtained from **5** by a direct, one-step, *bis*-amidation using the corresponding dicarboxylic acid chlorides and cyclization (Scheme 1), the shorter chain analogues were not amenable to this method because of intramolecular cyclization. Reaction of anhydrides with **1** yielded the monocarboxylic imidazoquinolines **2a** and **2b**; these intermediates were taken forward without purification and reacted with **1** to afford the *des*-amino **3** precursors. The amines at C4 and C4' were introduced by the protocol described in Chapter 2.

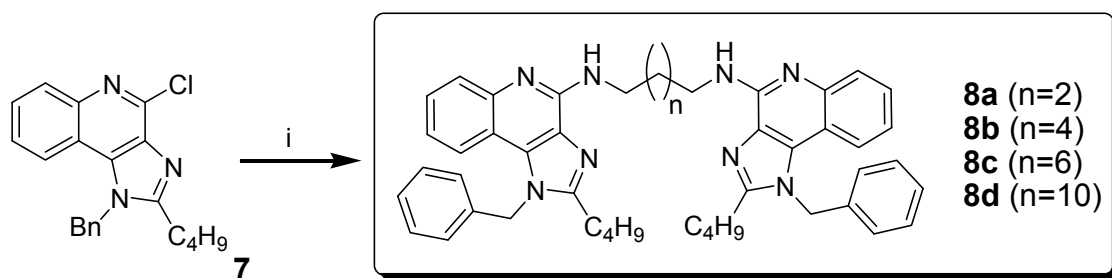
**Scheme 1.** Syntheses of imidazoquinoline dimers linked at C2.



Reagents: Glutaric anhydride (n=1) or adipic anhydride (n=2), Et<sub>3</sub>N, THF, 110 °C; ii. **1**, HBTU, Et<sub>3</sub>N, DMAP, DMF, 90 °C; iii. (a) 3-Chloroperoxybenzoic acid, CH<sub>2</sub>Cl<sub>2</sub>, CHCl<sub>3</sub>, MeOH, 45 °C; (b) Benzoyl isocyanate, CH<sub>2</sub>Cl<sub>2</sub>, 45 °C; (c) NaOCH<sub>3</sub>, MeOH, 80 °C; iv. (a) Suberoyl chloride (n=4) or dodecanedioyl dichloride (n=8), Et<sub>3</sub>N, THF; (b) NH<sub>3</sub>/MeOH, 150 °C.

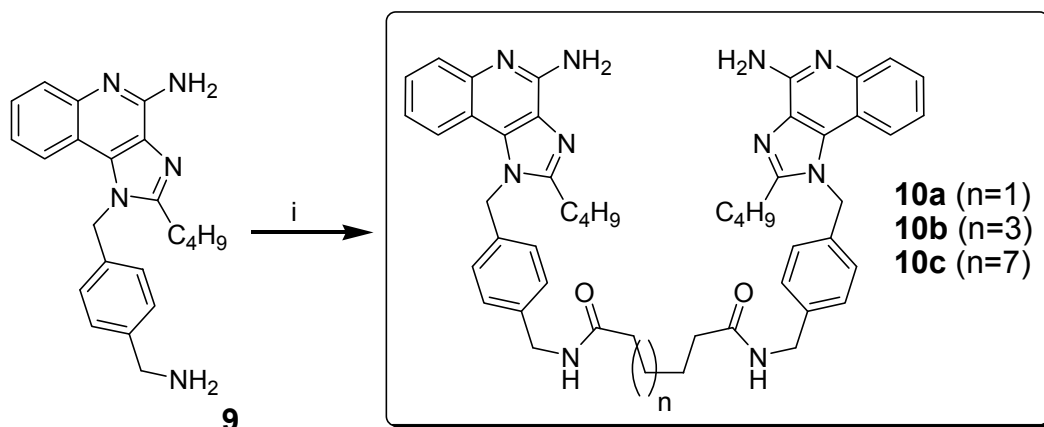
Next, the dimers linked via the C4-NH<sub>2</sub> (**8a-d**) were synthesized by direct S<sub>N</sub>Ar on **7** using  $\alpha,\omega$ -bis-amino alkanes (Scheme 2). Similarly, dimers linked at the N<sup>1</sup> position on the 4-aminomethylene benzyl group (**10a-c**) were obtained using appropriate dicarboxylic acid chlorides (Scheme 3).

**Scheme 2.** Syntheses of imidazoquinoline dimers linked at C4-NH<sub>2</sub>.



Reagents: i. 1,4-Diaminobutane (n=2) or 1,8-diaminooctane (n=4) or 1,10-diaminodecane (n=6) or 1,12-diaminododecane (n=10), MeOH, 140 °C;

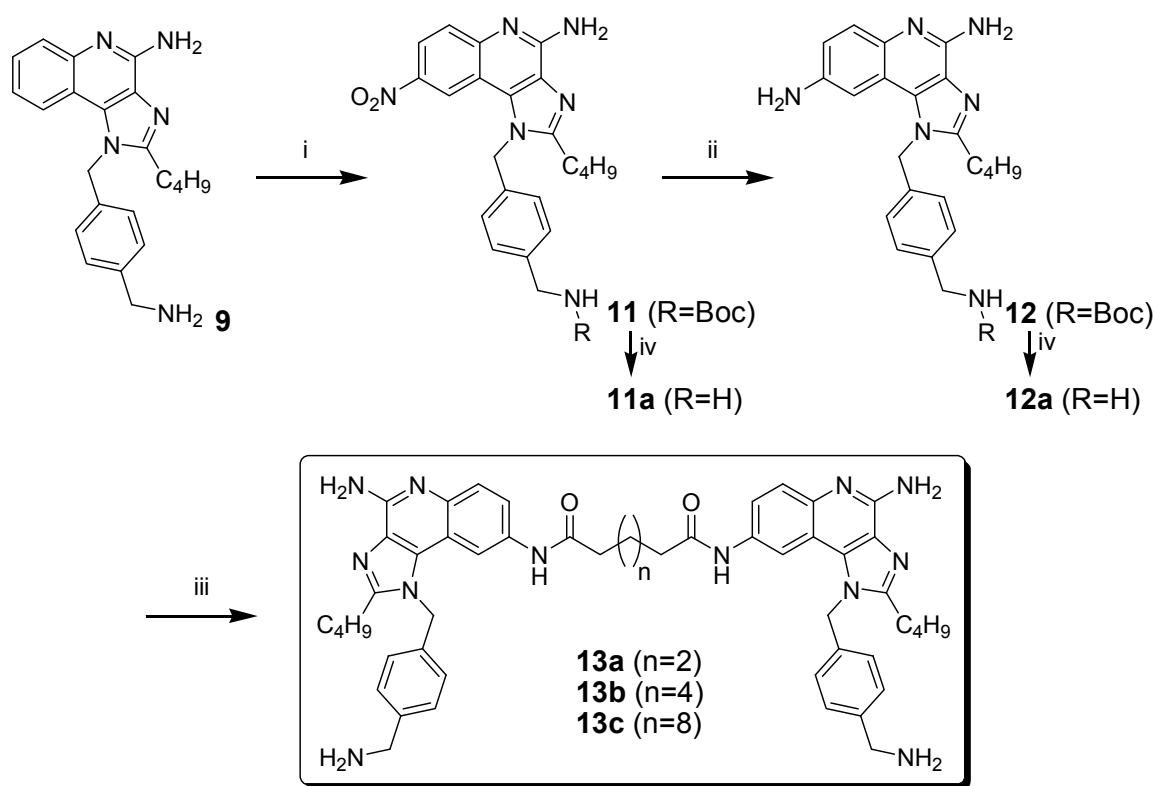
**Scheme 3.** Syntheses of imidazoquinoline dimers linked at N<sup>1</sup>-(4-aminomethylene)benzyl.



i. Adipoyl chloride (n=1) or suberoyl chloride (n=3) or dodecanedioyl dichloride (n=7), Et<sub>3</sub>N, THF.

Linking the **13** series of dimers via the quinoline ring required the introduction of an additional amine at position C8. This was achieved via carefully controlled nitration of **9** using 1.2-1.3 equiv. of HNO<sub>3</sub>, followed by *N*-Boc protection of the amine on the *N*<sup>1</sup> substituent, and subsequent reduction. Dimerization of the 4,8-diaminoimidazoquinoline **12** proceeded smoothly using dicarboxylic acid chlorides as described in the previous schemes. It is to be noted that the mono-nitro and mono-amino precursors **11** and **12** were *N*-Boc deprotected and tested for TLR-modulatory activities (see below, Table 1).

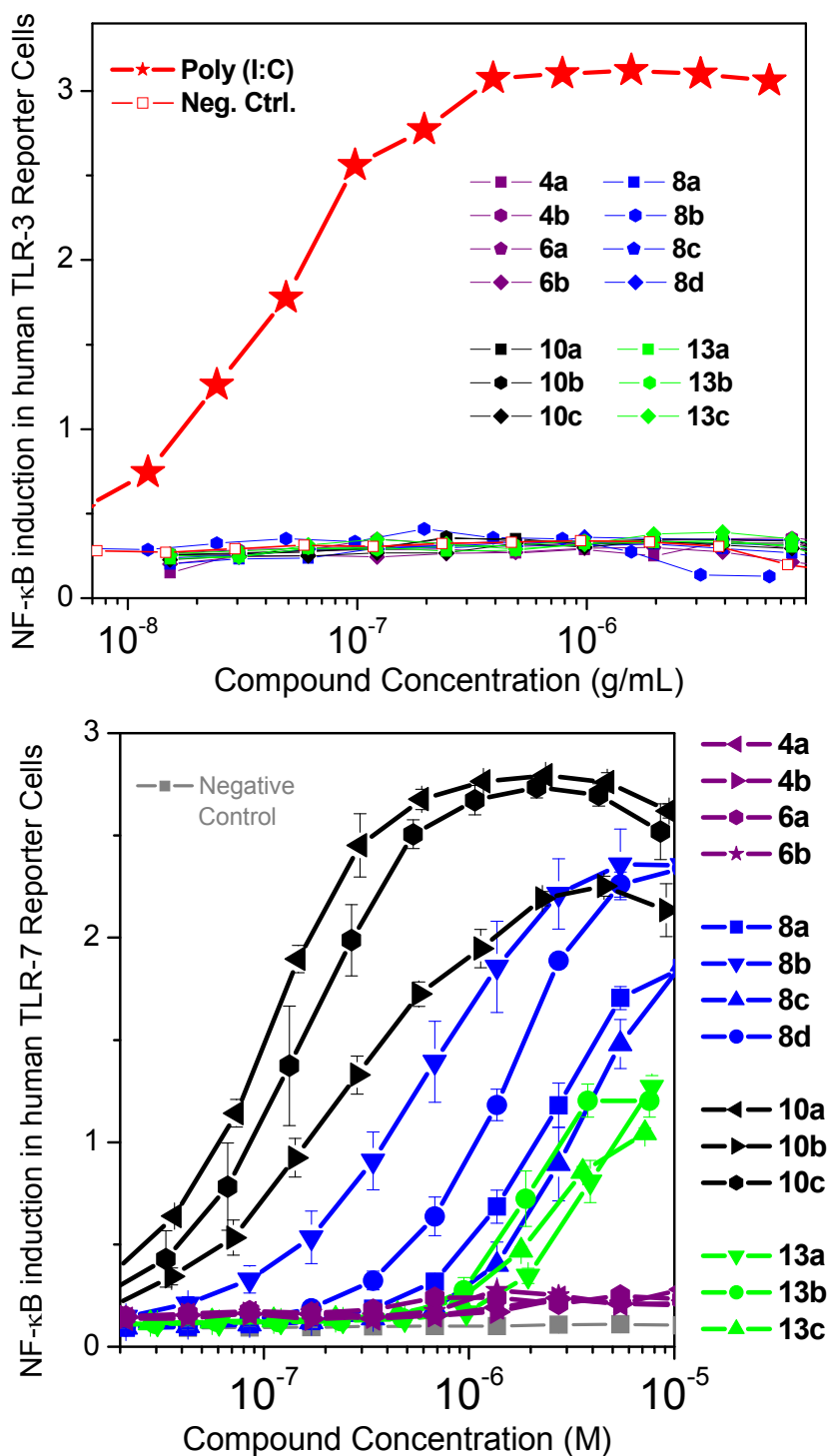
**Scheme 4.** Syntheses of imidazoquinoline dimers linked at C8-NH<sub>2</sub>.



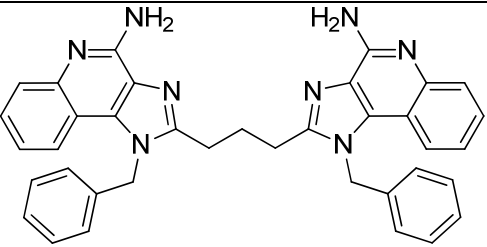
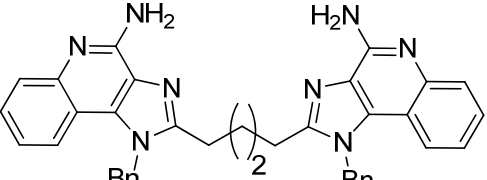
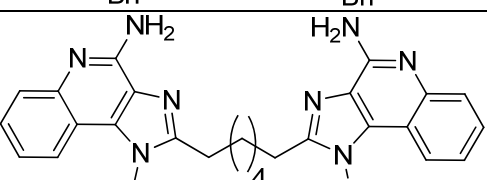
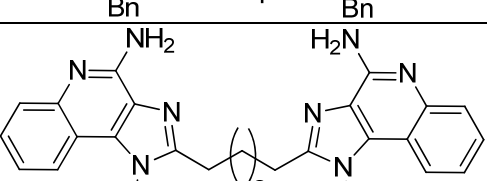
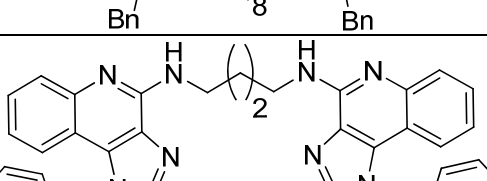
Reagents: (a) HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>; (b) (Boc)<sub>2</sub>O, Et<sub>3</sub>N, MeOH; ii. H<sub>2</sub>, Pt/C, MeOH, 60 psi; iii. (a) Adipoyl chloride (n=2) or suberoyl chloride (n=4) or dodecanedioyl dichloride (n=8), Et<sub>3</sub>N, THF; (b) HCl/dioxane, iv. HCl/dioxane.

All compounds were unfortunately found to be inactive in TLR3 reporter gene assays (**Fig. 3**). However, with the exception of **4** and **6** series of compounds, all other dimers retained TLR7-agonistic properties, the **10** series being the most potent (Fig. 4, Table 1).

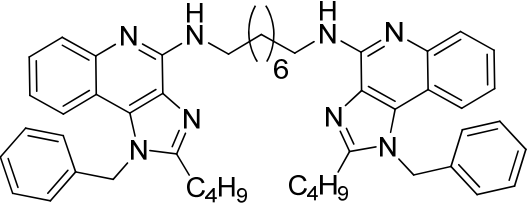
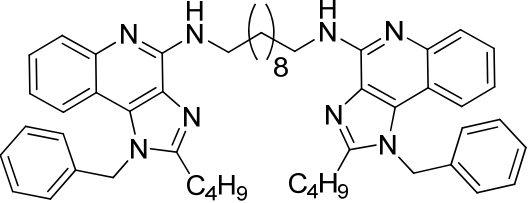
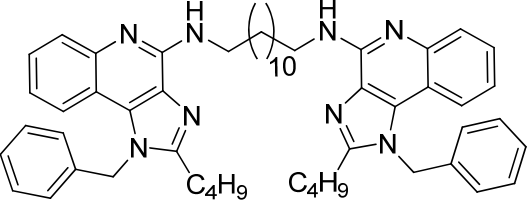
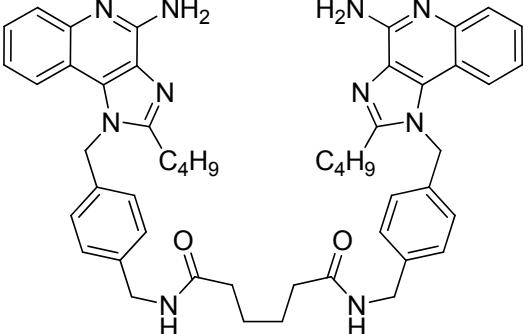
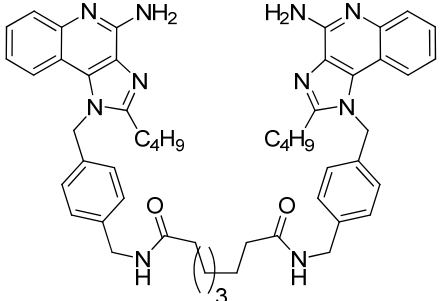
*Fig. 3. TLR3 (Top) and TLR7 (bottom) agonistic activities of the imidazoquinoline dimers.*



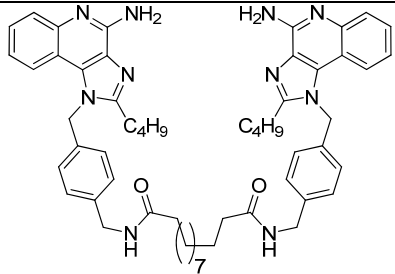
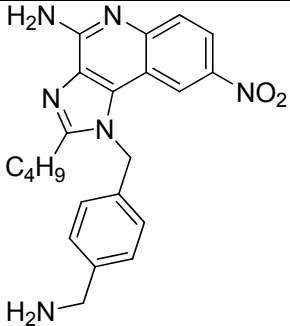
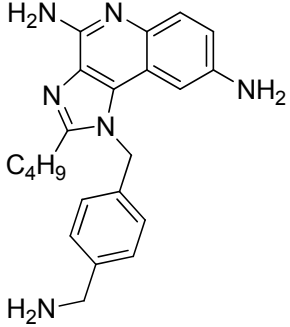
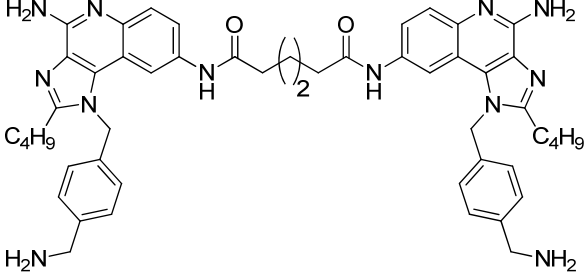
**Table 1.** Agonistic and antagonistic activities of the dimers in TLR3, TLR7, and TLR8 reporter gene assays. ND= not detected; NT=not tested.

Compound number	Structure	TLR3		TLR7		TLR8	
		Agonism (μM)	Antagonism (μM)	Agonism (μM)	Antagonism (μM)	Agonism (μM)	Antagonism (μM)
4a		ND	ND	ND	0.65	ND	3.2
4b		ND	ND	ND	1.45	ND	15.63
6a		ND	ND	ND	ND	ND	10.92
6b		ND	ND	ND	17.88	ND	4.65
8a		ND	ND	2.05	ND	ND	ND

**Table 1 (contd).** Agonistic and antagonistic activities of the dimers in TLR3, TLR7, and TLR8 reporter gene assays. ND= not detected; NT=not tested.

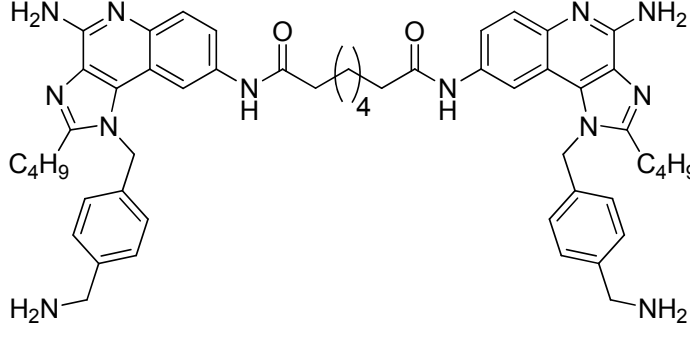
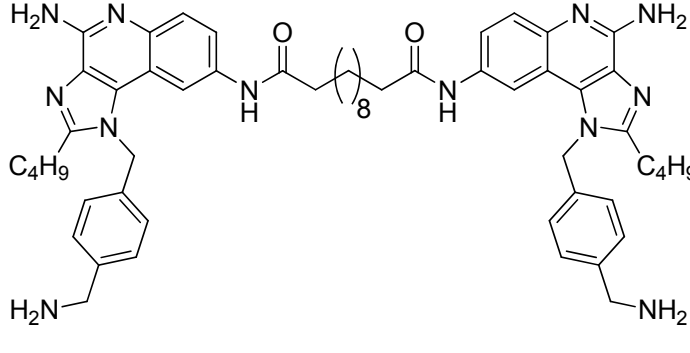
<b>8b</b>		ND	ND	0.56	ND	ND	ND
<b>8c</b>		ND	ND	3.00	ND	ND	ND
<b>8d</b>		ND	ND	1.42	ND	ND	ND
<b>10a</b>		ND	ND	0.11	ND	ND	ND
<b>10b</b>		ND	ND	0.24	ND	ND	ND

**Table 1 (contd).** Agonistic and antagonistic activities of the dimers in TLR3, TLR7, and TLR8 reporter gene assays. ND= not detected; NT=not tested.

<b>10c</b>		ND	ND	0.17	ND	4.78	ND
<b>11a</b>		NT	NT	0.56	ND	ND	ND
<b>12a</b>		NT	NT	0.45	ND	ND	ND
<b>13a</b>		ND	ND	7.24	ND	ND	ND



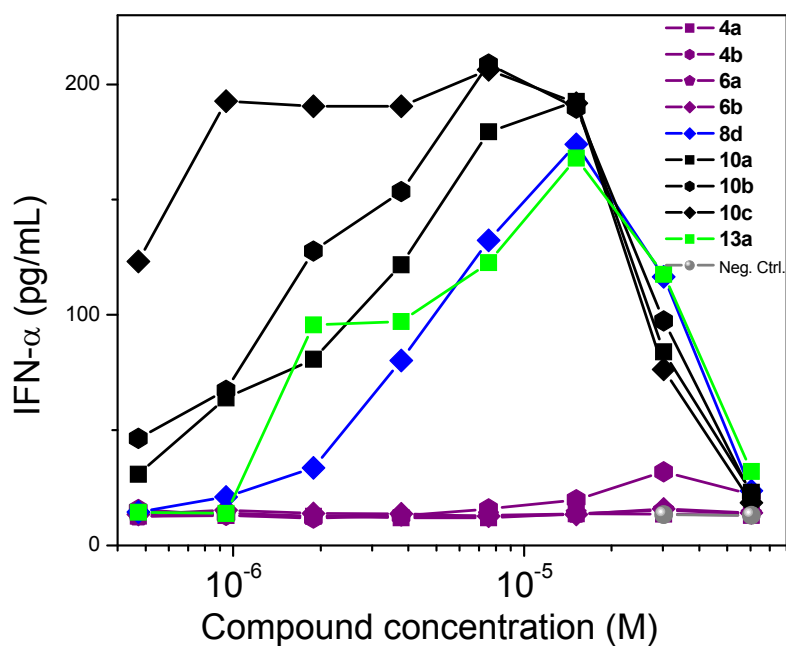
**Table 1 (contd).** Agonistic and antagonistic activities of the dimers in TLR3, TLR7, and TLR8 reporter gene assays. ND= not detected; NT=not tested.

<b>13b</b>		ND	ND	4.02	ND	ND	ND
<b>13c</b>		ND	ND	5.4	ND	ND	ND

The C2-liked dimeric compounds **4a**, **4b**, **6a**, and **6b** unexpectedly showed antagonistic activity in both TLR7 and TLR8 assays, with **4a** being most potent (0.65 and 3.2  $\mu\text{M}$   $\text{IC}_{50}$  values in TLR7 and TLR8 assays, respectively; Table 1). As discussed in Chapter 3, TLR7- and TLR8 antagonists are being actively pursued as therapeutic leads in HIV and autoimmune diseases, and this may be yet another chemotype worth following up.

As mentioned in the Introduction to this Chapter, ligation of TLR7 and TLR8 trigger inflammatory responses characterized by the elaboration of type I interferon ( $\text{IFN-}\alpha/\beta$ ) by virus-infected cells via activation of downstream NF- $\kappa\text{B}$  and IFN- $\beta$  promoters.<sup>182-187</sup> IFN production is a hallmark response underlying cellular antiviral immune responses. It was desirable to verify that TLR7 agonism that we had observed (**Fig. 3**) manifested in IFN production in secondary screens. Using an *ex vivo* stimulation model using human peripheral blood mononuclear cells (hPBMC), it was demonstrated that IFN- $\alpha$  was indeed induced in a dose-dependent, bimodal manner as expected for innate immune responses (**Fig. 4**). Compound **10c** was found to be the most potent. The **4** and **6** series were quiescent (**Fig. 4**), consistent with their apparent antagonistic behavior.

**Fig. 4.** IFN- $\alpha$  induction by select dimers in human peripheral blood mononuclear cells. IFN- $\alpha$  was assayed by analyte specific ELISA after incubation of hPBMC with graded concentrations of the test compound for 12h.



Despite the significant differences in physicochemical (and, structural) properties, several of the dimeric compounds seemed indistinguishable from the parent imidazoquinolines (**31** in Chapters 4 and **5d** in Chapter 5) in terms of their TLR7-agonistic profiles in primary and secondary screens. In order to ensure that we were not overlooking possibly subtle differences, we performed transcriptomal analyses on **10c** (Table 2). As expected, **10c** strongly upregulated Type I IFN; however, the most prominent upregulated gene was IFN- $\omega$  (Table 2). A comparison of Type IFN- $\alpha/\beta$  and IFN- $\omega$  transcriptional induction by these compounds show a very prominent inversion of IFN- $\alpha/\beta$  and IFN- $\omega$  transcript ratios for **10c**. A single functional gene in the human genome codes for IFN- $\omega$ , a monomeric glycoprotein distantly related in structure to IFN- $\alpha$  and IFN- $\beta$ , but unrelated to IFN- $\gamma$ .<sup>201-203</sup>

Chronic hepatitis C (HCV) affects 2.2 - 3% of the world population. Pegylated IFN- $\alpha$  in combination with ribavirin, is the standard of care, leading to viral eradication in only about 50% of treated patients. The use of IFN- $\alpha$  is associated with severe side effects, and endogenous Type I IFN inducers<sup>204-208</sup> (such as the imidazoquinolines and 8-oxo-3-deazapurines) are being investigated intensively as less-expensive, and orally available therapeutic alternatives. In addition to prominent IFN- $\alpha$  induction (**Fig. 4**), **10c** strongly induces IFN- $\omega$  transcripts (**Fig. 5**), and whether this is paralleled by IFN- $\omega$  protein secretion is yet to be verified.

The response of hepatitis C virus to IFN- $\alpha$  and ribavirin is known to be genotype-specific, and HCV genotype IV is known to respond poorly to IFN- $\alpha$ .<sup>209</sup> In patients resistant to therapy with conventional IFN- $\alpha$ /ribavirin therapy, IFN- $\omega$  may be of value,<sup>210-213</sup> and clinical trials are currently underway. A synthetically accessible, orally bioavailable, small-molecule inducer of endogenous IFN- $\alpha$  and IFN- $\omega$  may therefore be of considerable practical value, and we are currently evaluating this compound in detail.

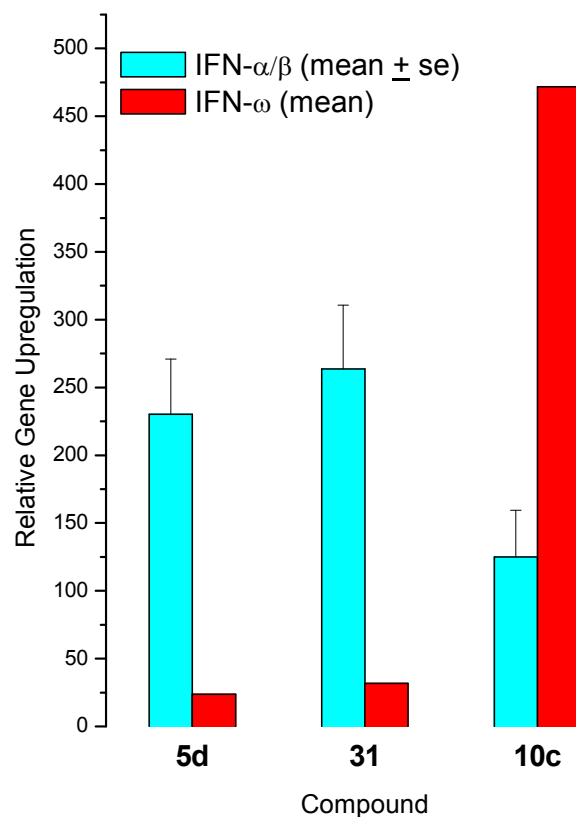
**Table 2.** Top 50 transcripts upregulated in human PBMCs by **10c** as analyzed by transcriptomal profiling.

Probe set ID	GeneSymbol	GeneName	Fold change
207817_at	IFNW1	interferon, omega 1	471.72
208173_at	IFNB1	interferon, beta 1, fibroblast	375.55
204533_at	CXCL10	chemokine (C-X-C motif) ligand 10	374.66
	IFNA1 ///		
208344_x_at	IFNA13	interferon, alpha 1 /// interferon, alpha 13	358.54
208075_s_at	CCL7	chemokine (C-C motif) ligand 7	345.69
208375_at	IFNA1	interferon, alpha 1	293.33
209774_x_at	CXCL2	chemokine (C-X-C motif) ligand 2	227.91
208259_x_at	IFNA7	interferon, alpha 7	182.19
210163_at	CXCL11	chemokine (C-X-C motif) ligand 11	175.39
211506_s_at	IL8	interleukin 8	171.5
205476_at	CCL20	chemokine (C-C motif) ligand 20	158.03
211338_at	IFNA2	interferon, alpha 2	150.95
205207_at	IL6	interleukin 6 (interferon, beta 2)	99.41
206025_s_at	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	87.87
207932_at	IFNA8	interferon, alpha 8	87.3
214569_at	IFNA5	interferon, alpha 5	85.58
211122_s_at	CXCL11	chemokine (C-X-C motif) ligand 11	85.49
1554997_a_at	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	84.86
207194_s_at	ICAM4	intercellular adhesion molecule 4 (Landsteiner-Wiener blood group)	81.11
204748_at	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	78.96
214038_at	CCL8	chemokine (C-C motif) ligand 8	78.62
207850_at	CXCL3	chemokine (C-X-C motif) ligand 3	75.85
	CCL3 ///		
	CCL3L1 ///		
	CCL3L3 ///		
205114_s_at	LOC728830	chemokine (C-C motif) ligand 3 /// chemokine (C-C motif) ligand 3-like 1 /// chemokine (C-C motif) ligand 3-like 3 /// similar to C-C motif chemokine 3-like 1 precursor (Small-inducible cytokine A3-like 1) (Tonsillar lymphocyte LD78 beta protein) (LD78-be	75.65
216598_s_at	CCL2	chemokine (C-C motif) ligand 2	74.27
208548_at	IFNA6	interferon, alpha 6	73.63
204103_at	CCL4	chemokine (C-C motif) ligand 4	72.7
210118_s_at	IL1A	interleukin 1, alpha	71.52
203153_at	IFIT1	interferon-induced protein with tetratricopeptide repeats 1	70.62
206026_s_at	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	64.93
217502_at	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	62.04
204747_at	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	60.1
205067_at	IL1B	interleukin 1, beta	58.33
206157_at	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	58.25
213797_at	RSAD2	radical S-adenosyl methionine domain containing 2	57.87
242625_at	RSAD2	radical S-adenosyl methionine domain containing 2	54.35
207113_s_at	TNF	tumor necrosis factor (TNF superfamily, member 2)	53.29

39402_at	IL1B	interleukin 1, beta	51.61
216243_s_at	IL1RN	interleukin 1 receptor antagonist	49.11
229450_at	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	46.83
212657_s_at	IL1RN	interleukin 1 receptor antagonist	43.61
228377_at	KLHL14	kelch-like 14 (Drosophila)	42.45
202859_x_at	IL8	interleukin 8	42.25
204363_at	F3	coagulation factor III (thromboplastin, tissue factor)	40.5
229437_at	MIRHG2	microRNA host gene 2 (non-protein coding)	39.87
1552609_s_at	IL28A /// IL28B	interleukin 28A (interferon, lambda 2) /// interleukin 28B (interferon, lambda 3)	39.49
205306_x_at	KMO	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	37.06
240287_at	IRG1	immunoresponsive 1 homolog (mouse)	36.46
232504_at	LOC285628	hypothetical protein LOC285628	35.64
205767_at	EREG	epiregulin	34.7
211138_s_at	KMO	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	32.37
204667_at	FOXA1	forkhead box A1	30.84
204614_at	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	30.66
214438_at	HLX	H2.0-like homeobox	28.34
208182_x_at	IFNA14	interferon, alpha 14	27.25
206765_at	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	26.81
208448_x_at	IFNA16	interferon, alpha 16	26.55
204803_s_at	RRAD	Ras-related associated with diabetes	24.53
211405_x_at	IFNA17	interferon, alpha 17	24.38
213524_s_at	G0S2	G0/G1switch 2	24.38
218943_s_at	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	24.19
207964_x_at	IFNA4	interferon, alpha 4	23.98
216016_at	NLRP3	NLR family, pyrin domain containing 3	23.83

**Fig. 5.** Left: Relative IFN- $\alpha/\beta$  and IFN- $\omega$  gene upregulation in hPBMCs as determined by transcriptomal profiling. A concentration of 10  $\mu$ M of compound and  $10^7$  PBMCs/sample with 6 h incubation was used. Right: graph showing inversion of IFN- $\alpha/\beta$  and IFN- $\omega$  transcript ratios for **10c**.

Gene Symbol	5d (Chapter 5)	31 (Chapter 4)	10c
IFNA1	243.22	319.58	293.33
IFNA2	401.02	457.90	150.95
IFNA4	182.68	179.17	23.98
IFNA5	246.43	372.99	85.58
IFNA6	71.46	62.91	73.63
IFNA7	163.80	174.59	182.19
IFNA8	144.11	151.51	87.3
IFNA10	236.93	270.17	23.18
IFNA1 /// IFNA13	141.41	152.78	358.54
IFNA14	297.30	339.48	27.25
IFNA16	669.73	739.23	26.55
IFNA17	108.96	113.17	24.38
IFNA21	161.56	168.01	18.66
IFNB1	156.30	190.91	375.55
IFNG	17.82	5.87	11.21
IFNW1	23.71	31.85	471.72



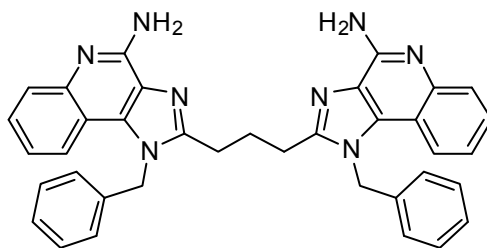
### 6.3. Conclusions.

'Homodimeric' imidazoquinoline constructs connected by polymethylene units of varying lengths at C2, C4-NH<sub>2</sub>, *N*<sup>1</sup>-(4-aminomethylene)benzyl and C8-NH<sub>2</sub> have been synthesized to test whether any of such dimeric molecules would modulate (agonize or antagonize) TLR3 activities. No TLR3-antagonistic activities were detected in any of the compounds. However, TLR7 and TLR8 dual-antagonism was found in C2-linked dimers, typified by **4a**. IFN- $\alpha$  and IFN- $\omega$ -inducing activities were found in the **10** series, with the most potent compound being **10c**. These compounds serve as good leads for developing compounds for suppressing autoimmune phenomena (**4a**) and inducing endogenous IFNs for the treatment of hepatitis (**10c**).

#### 6.4. Experimental.

All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instrument unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm x 150 mm, 5 µm analytical reverse phase C<sub>18</sub> column with H<sub>2</sub>O-isopropanol or H<sub>2</sub>O-CH<sub>3</sub>CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion acquisition mode. All the compounds synthesized were obtained as solids.

**Synthesis of Compound 4a: 2,2'-(propane-1,3-diyl)bis(1-benzyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine).**

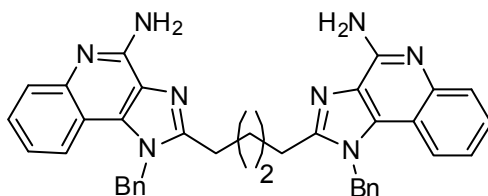


To a solution of **1** (100 mg, 0.4 mmol) in anhydrous THF, were added triethylamine (53 mg, 0.52 mmol) and glutaric anhydride (60 mg, 0.52 mmol) and the reaction vessel was heated in a microwave for 2 hours at 110 °C. The solvent was then removed under vacuum to obtain the crude product **2**, which was then dissolved in anhydrous DMF and to this solution, were added HBTU (167 mg, 0.44 mmol), triethylamine (53 mg, 0.52 mmol), **1** (100 mg, 0.4 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred for 12 hours at 90 °C. The solvent was then removed under

vacuum and the residue was purified using column chromatography (12% MeOH/dichloromethane) to obtain the intermediate the compound **3** (157 mg). To a solution of **3** in solvent mixture of MeOH:dichloromethane:chloroform (0.1:1:1), was added 3-chloroperoxybenzoic acid (242 mg, 1.4 mmol) and the reaction mixture was refluxed at 45 °C for 40 minutes. The solvent was then removed and the residue was purified using column chromatography (35 % MeOH/dichloromethane) to obtain the *bis-N*-oxide derivative (130 mg). *bis-N*-oxide derivative (110 mg, 0.19 mmol) was then dissolved in anhydrous dichloromethane, followed by addition of benzoyl isocyanate (96 mg, 0.67 mmol) and heated at 45 °C for 15 minutes. The solvent was then removed under vacuum and the residue was dissolved in anhydrous MeOH, followed by addition of excess of sodium methoxide and heated at 80 °C for 2 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (50% MeOH/dichloromethane) to obtain the compound **2** (25 mg, 11%). <sup>1</sup>H NMR (500 MHz, DMSO) δ 14.30 (s, 2H), 9.48 – 8.30 (bs, 4H), 7.93 (d, *J* = 8.3 Hz, 2H), 7.77 (d, *J* = 8.3 Hz, 2H), 7.65 – 7.60 (m, 2H), 7.38 – 7.34 (m, 2H), 7.26 (t, *J* = 7.6 Hz, 4H), 7.17 (t, *J* = 7.4 Hz, 2H), 7.03 (d, *J* = 7.4 Hz, 4H), 5.94 (s, 4H), 3.16 (t, *J* = 7.2 Hz, 4H), 2.44 – 2.35 (m, 2H). <sup>13</sup>C NMR (126 MHz, DMSO) δ 156.22, 148.86, 135.32, 135.30, 133.48, 129.51, 128.93, 127.57, 125.47, 124.72, 124.54, 121.49, 118.31, 112.16, 48.35, 25.21, 24.43. MS (ESI) calculated for C<sub>37</sub>H<sub>32</sub>N<sub>8</sub>, *m/z* 588.2750, found 589.2860 (*M* + H)<sup>+</sup>.

**Compound 4b was synthesized similarly as described for compound 4a.**

**Synthesis of Compound 4b: 2,2'-(butane-1,4-diyl)*bis*(1-benzyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine).**

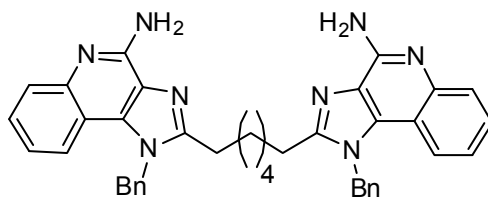


<sup>1</sup>H NMR (500 MHz, DMSO) δ 13.86 (s, 2H), 8.88 (bs, 4H), 7.93 (d, *J* = 8.2 Hz, 2H), 7.83 – 7.79 (m, 2H), 7.66 – 7.61 (m, 2H), 7.39 – 7.34 (m, 2H), 7.27 (t, *J* = 7.6 Hz, 4H), 7.18 (t, *J* = 7.4 Hz, 2H), 7.01



(d,  $J = 7.4$  Hz, 4H), 5.94 (s, 4H), 2.99 (s, 4H), 1.83 (s, 4H).  $^{13}\text{C}$  NMR (126 MHz, DMSO)  $\delta$  156.55, 148.75, 135.38, 133.62, 129.50, 128.95, 127.60, 125.42, 124.74, 124.54, 121.54, 118.48, 112.28, 48.34, 26.49, 26.14. MS (ESI) calculated for  $\text{C}_{38}\text{H}_{34}\text{N}_8$ ,  $m/z$  602.2906, found 603.3272 ( $\text{M} + \text{H}$ ) $^+$  and 302.1705 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

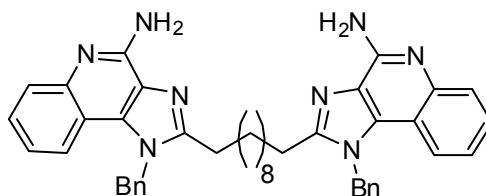
**Synthesis of Compound 6a:** 2,2'-(hexane-1,6-diyl)bis(1-benzyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine).



To a solution of **5** (60 mg, 0.21 mmol) in anhydrous THF, were added triethylamine (54 mg, 0.53 mmol), and suberoyl chloride (23 mg, 0.11 mmol) and the reaction mixture was stirred for 6 hours. The solvent was then removed under vacuum and the residue was dissolved in EtOAc and washed with water/brine. The EtOAc fraction was then dried using sodium sulfate and evaporated under vacuum to obtain the intermediate amide compound, which was then dissolved in 1 mL solution of 2M ammonia in MeOH and heated at 150 °C for 15 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (20% MeOH/dichloromethane) to obtain the compound **6a** (8 mg, 12 %).  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  7.96 (d,  $J = 8.3$  Hz, 2H), 7.77 (d,  $J = 8.4$  Hz, 2H), 7.65 (dd,  $J = 11.5, 4.2$  Hz, 2H), 7.39 (t,  $J = 7.8$  Hz, 2H), 7.31 (t,  $J = 7.4$  Hz, 4H), 7.25 (t,  $J = 7.3$  Hz, 2H), 7.06 (d,  $J = 7.4$  Hz, 4H), 5.93 (s, 4H), 2.97 (t,  $J = 7.5$  Hz, 4H), 1.85 (d,  $J = 7.0$  Hz, 4H), 1.43 (s, 4H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  158.93, 137.73, 136.20, 135.30, 131.11, 130.47, 129.31, 126.65, 126.57, 125.81, 122.94, 119.65, 114.20, 50.13, 29.70, 27.92. MS (ESI) calculated for  $\text{C}_{40}\text{H}_{38}\text{N}_8$ ,  $m/z$  630.3219, found 631.3415 ( $\text{M} + \text{H}$ ) $^+$ .

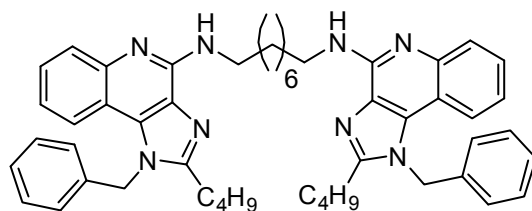
**Compound 6b** was synthesized similarly as described for compound **6a**.

**Synthesis of Compound 6b: 2,2'-(decane-1,10-diyl)bis(1-benzyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine).**



$^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  7.86 (d,  $J$  = 8.3 Hz, 2H), 7.69 (d,  $J$  = 8.4 Hz, 2H), 7.51 (t,  $J$  = 7.7 Hz, 2H), 7.32 (t,  $J$  = 7.3 Hz, 4H), 7.28 (d,  $J$  = 7.2 Hz, 2H), 7.23 (t,  $J$  = 7.7 Hz, 2H), 7.06 (d,  $J$  = 7.4 Hz, 4H), 5.88 (s, 4H), 2.96 (t,  $J$  = 7.6 Hz, 4H), 1.79 (dt,  $J$  = 15.3, 7.7 Hz, 4H), 1.37 (dd,  $J$  = 14.9, 7.4 Hz, 4H), 1.32 – 1.24 (m,  $J$  = 11.6 Hz, 4H), 1.23 (d,  $J$  = 10.1 Hz, 4H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  157.44, 151.60, 136.61, 130.41, 129.74, 129.22, 126.69, 126.46, 124.90, 123.38, 122.15, 115.12, 50.05, 30.32, 30.25, 30.17, 28.49, 28.17. MS (ESI) calculated for  $\text{C}_{44}\text{H}_{46}\text{N}_8$ ,  $m/z$  686.3845, found 687.3749 ( $\text{M} + \text{H}$ ) $^+$  and 344.1949 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

**Synthesis of Compound 8b:  $N^1, N^8$ -bis(1-benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-yl)octane-1,8-diamine.**

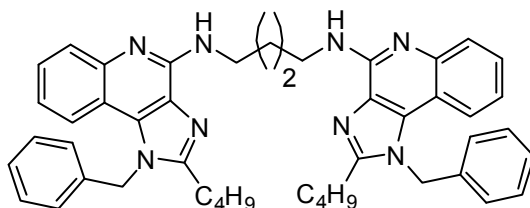


To a solution of **7** (50 mg, 0.14 mmol) in 1 mL of anhydrous MeOH, was added 1,8-diaminooctane (10 mg, 0.07 mmol) and the reaction mixture was heated at 140 °C for 4 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (8% MeOH/dichloromethane) to obtain the compound **8b** (12 mg, 22%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.87 (d,  $J$  = 8.3 Hz, 2H), 7.68 (d,  $J$  = 8.2 Hz, 2H), 7.41 (t,  $J$  = 7.7 Hz, 2H), 7.36 – 7.26 (m, 6H), 7.09 – 7.01 (m, 6H), 5.78 (s, 2H), 5.72 (s, 4H), 3.77 (dd,  $J$  = 12.8, 6.6 Hz, 4H), 2.92 – 2.83 (m, 4H), 1.87 – 1.74 (m, 8H), 1.58 – 1.50 (m, 4H), 1.45 (dt,  $J$  = 15.1, 7.5 Hz, 8H), 0.93 (t,  $J$  = 7.4 Hz, 6H).  $^{13}\text{C}$  NMR

(101 MHz, CDCl<sub>3</sub>)  $\delta$  153.34, 150.78, 145.51, 135.62, 129.20, 127.93, 127.41, 127.07, 126.68, 125.59, 121.34, 119.52, 114.87, 48.81, 40.76, 30.18, 29.98, 29.50, 27.21, 22.56, 13.77. MS (ESI) calculated for C<sub>50</sub>H<sub>58</sub>N<sub>8</sub>, m/z 770.4784, found 771.4963 (M + H)<sup>+</sup> and 386.2570 (M + 2H)<sup>2+</sup>.

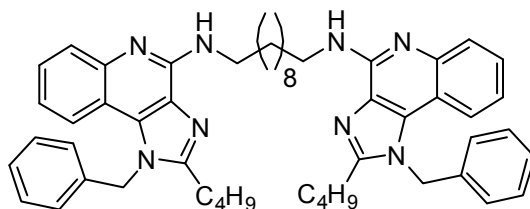
Compounds 8a, 8c and 8d were synthesized similarly as described for compound 8b.

**Synthesis of Compound 8a:** *N*<sup>1</sup>,*N*<sup>4</sup>-bis(1-benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-yl)butane-1,4-diamine.



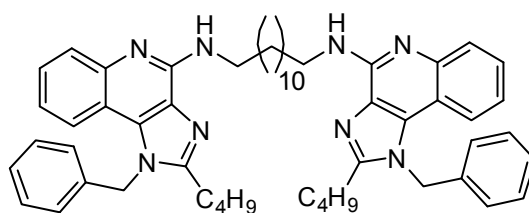
<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  7.86 (d, *J* = 8.2 Hz, 2H), 7.65 (dd, *J* = 8.2, 1.0 Hz, 2H), 7.41 – 7.35 (m, 2H), 7.35 – 7.24 (m, 6H), 7.09 – 6.99 (m, 6H), 5.86 (s, 2H), 5.69 (s, 4H), 3.87 (s, 4H), 2.88 – 2.81 (m, 4H), 2.00 (s, 4H), 1.76 (ddd, *J* = 13.0, 9.0, 7.7 Hz, 4H), 1.46 – 1.37 (m, 4H), 0.90 (t, *J* = 7.4 Hz, 6H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>)  $\delta$  151.27, 148.66, 143.44, 133.52, 130.85, 128.77, 127.28, 127.13, 125.85, 125.38, 124.95, 124.60, 123.50, 123.40, 119.29, 117.45, 112.81, 46.71, 38.40, 28.01, 25.51, 25.10, 20.47, 11.68. MS (ESI) calculated for C<sub>46</sub>H<sub>50</sub>N<sub>8</sub>, m/z 714.4158, found 715.4333 (M + H)<sup>+</sup> and 358.2263 (M + 2H)<sup>2+</sup>.

**Synthesis of Compound 8c:** *N*<sup>1</sup>,*N*<sup>10</sup>-bis(1-benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-yl)decane-1,10-diamine.



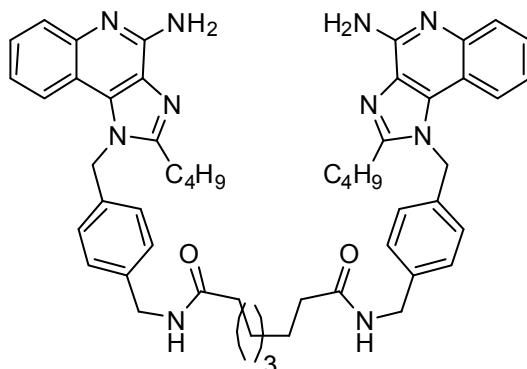
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.84 (d,  $J$  = 8.2 Hz, 2H), 7.66 (dd,  $J$  = 8.2, 1.0 Hz, 2H), 7.40 – 7.36 (m, 2H), 7.33 – 7.26 (m, 6H), 7.07 – 7.01 (m, 6H), 5.73 (s, 2H), 5.70 (s, 4H), 3.74 (dd,  $J$  = 12.7, 6.5 Hz, 4H), 2.88 – 2.83 (m, 4H), 1.80 – 1.72 (m, 8H), 1.53 – 1.24 (m, 16H), 0.91 (t,  $J$  = 7.4 Hz, 6H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  151.76, 149.23, 143.97, 134.05, 131.32, 127.81, 127.63, 126.36, 125.85, 125.50, 125.10, 124.01, 123.84, 119.75, 117.95, 113.30, 47.23, 39.17, 28.62, 28.39, 28.06, 27.95, 25.65, 20.98, 12.20. MS (ESI) calculated for  $\text{C}_{52}\text{H}_{62}\text{N}_8$ ,  $m/z$  798.5097, found 799.5416 ( $\text{M} + \text{H}$ ) $^+$  and 400.2799 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

**Synthesis of Compound 8d:**  $N',N'^{12}$ -bis(1-benzyl-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-yl)dodecane-1,12-diamine.



$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.84 (d,  $J$  = 8.2 Hz, 2H), 7.65 (dd,  $J$  = 8.2, 1.0 Hz, 2H), 7.38 (ddd,  $J$  = 8.3, 7.1, 1.3 Hz, 2H), 7.34 – 7.24 (m, 6H), 7.06 – 7.00 (m, 6H), 5.74 (s, 2H), 5.69 (s, 4H), 3.74 (dd,  $J$  = 12.6, 6.5 Hz, 4H), 2.86 (dd,  $J$  = 17.4, 9.6 Hz, 4H), 1.83 – 1.69 (m, 8H), 1.56 – 1.30 (m, 20H), 0.91 (t,  $J$  = 7.4 Hz, 6H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  151.81, 149.27, 144.02, 134.08, 131.36, 127.68, 126.41, 125.87, 125.53, 125.16, 124.05, 119.81, 118.00, 113.34, 47.27, 39.25, 28.65, 28.44, 28.15, 28.13, 28.01, 25.70, 25.68, 21.03, 12.24. MS (ESI) calculated for  $\text{C}_{54}\text{H}_{66}\text{N}_8$ ,  $m/z$  826.5410, found 827.5796 ( $\text{M} + \text{H}$ ) $^+$  and 414.2977 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

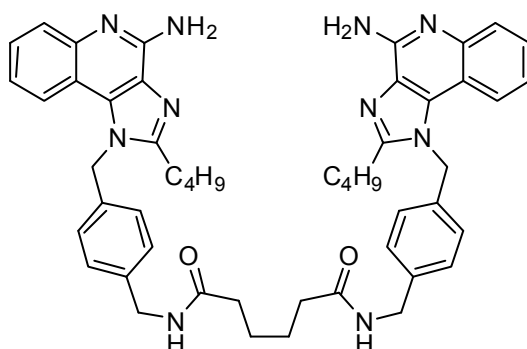
**Synthesis of Compound 10b:** *N*<sup>1</sup>,*N*<sup>8</sup>-bis(4-((4-amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzyl)octanediamide.



To a solution of **9** (25 mg, 0.058 mmol) in anhydrous THF, were added triethylamine (15 mg, 0.15 mmol) and suberoyl chloride (6 mg, 0.029 mmol). The reaction mixture was stirred for 1 hour and then the solvent was removed under vacuum. The residue was then purified using column chromatography (30% MeOH/dichloromethane) to obtain the compound **10b** (8 mg, 32%) <sup>1</sup>H NMR (500 MHz, MeOD) δ 7.65 (dd, *J* = 8.3, 0.9 Hz, 2H), 7.55 – 7.51 (m, 2H), 7.27 (ddd, *J* = 8.3, 7.1, 1.2 Hz, 2H), 7.12 (d, *J* = 8.2 Hz, 4H), 6.95 (ddd, *J* = 8.2, 7.2, 1.1 Hz, 2H), 6.88 (d, *J* = 8.2 Hz, 4H), 5.69 (s, 4H), 4.18 (s, 4H), 2.84 – 2.78 (m, 4H), 2.03 (t, *J* = 7.5 Hz, 4H), 1.64 (dt, *J* = 15.4, 7.6 Hz, 4H), 1.47 – 1.37 (m, 4H), 1.30 (dq, *J* = 14.8, 7.4 Hz, 4H), 1.16 – 1.10 (m, 4H), 0.80 (t, *J* = 7.4 Hz, 6H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 176.03, 156.18, 152.62, 144.89, 140.08, 136.09, 135.55, 129.44, 128.54, 126.95, 126.86, 126.21, 123.42, 121.58, 115.75, 49.58, 43.58, 36.85, 30.85, 29.75, 27.82, 26.74, 23.43, 14.11. MS (ESI) calculated for C<sub>52</sub>H<sub>60</sub>N<sub>10</sub>O<sub>2</sub>, *m/z* 856.4901, found 879.4711 (*M* + Na<sup>+</sup>) and 429.2430 (*M* + 2H)<sup>2+</sup>.

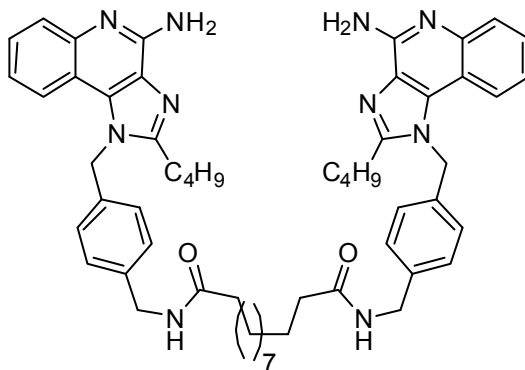
Compounds **10a** and **10c** were synthesized similarly as described for compound **10b**.

**Synthesis of Compound 10a:** *N*<sup>1</sup>,*N*<sup>8</sup>-bis(4-((4-amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzyl)adipamide.



$^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  7.85 (d,  $J$  = 7.6 Hz, 2H), 7.68 (d,  $J$  = 7.8 Hz, 2H), 7.52 – 7.45 (m, 2H), 7.26 (d,  $J$  = 8.2 Hz, 4H), 7.23 – 7.16 (m, 2H), 7.02 (d,  $J$  = 8.2 Hz, 4H), 5.85 (s, 4H), 4.30 (s, 4H), 2.99 – 2.93 (m, 4H), 2.19 (t,  $J$  = 6.0 Hz, 4H), 1.80 (dd,  $J$  = 15.3, 7.7 Hz, 4H), 1.58 (t,  $J$  = 3.1 Hz, 4H), 1.45 (dd,  $J$  = 15.0, 7.5 Hz, 4H), 0.94 (t,  $J$  = 7.4 Hz, 6H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  174.28, 156.00, 150.28, 138.78, 135.01, 134.27, 128.13, 128.05, 125.44, 123.28, 121.97, 120.73, 113.67, 48.28, 42.15, 35.17, 29.21, 26.38, 25.03, 21.96, 12.68. MS (ESI) calculated for  $\text{C}_{50}\text{H}_{56}\text{N}_{10}\text{O}_2$ ,  $m/z$  828.4588, found 829.4440 ( $\text{M} + \text{H}$ ) $^+$  and 415.2244 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

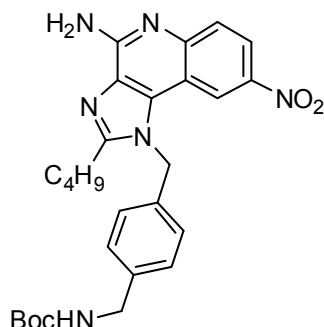
**Synthesis of Compound 10c:**  $N^1, N^{12}$ -bis(4-((4-amino-2-butyl-1H-imidazo[4,5-c]quinolin-1-yl)methyl)benzyl)dodecanediamide.



$^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  7.69 (dd,  $J$  = 8.3, 0.8 Hz, 2H), 7.54 (dd,  $J$  = 8.4, 0.7 Hz, 2H), 7.31 (ddd,  $J$  = 8.4, 7.2, 1.2 Hz, 2H), 7.14 (d,  $J$  = 8.2 Hz, 4H), 7.00 (ddd,  $J$  = 8.2, 7.2, 1.1 Hz, 2H), 6.90 (d,  $J$  = 8.2 Hz, 4H), 5.73 (s, 4H), 4.20 (s, 4H), 2.85 – 2.81 (m, 4H), 2.06 (t,  $J$  = 7.4 Hz, 4H), 1.66 (dt,  $J$  = 15.4, 7.6 Hz, 4H), 1.44 (dt,  $J$  = 14.4, 7.3 Hz, 4H), 1.31 (dq,  $J$  = 14.8, 7.4 Hz, 4H), 1.10 (dd,  $J$  = 29.4, 26.2 Hz, 12H), 0.81 (t,  $J$  = 7.4 Hz, 6H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  176.17, 156.64, 152.26, 143.34,

140.20, 135.93, 135.90, 129.44, 128.92, 126.85, 126.77, 125.13, 123.90, 121.81, 115.51, 49.64, 43.55, 36.99, 30.78, 30.36, 30.23, 30.11, 27.82, 26.96, 23.41, 14.11. MS (ESI) calculated for  $C_{56}H_{68}N_{10}O_2$ ,  $m/z$  912.5527, found 913.5886 ( $M + H$ )<sup>+</sup> and 457.2974 ( $M + 2H$ )<sup>2+</sup>.

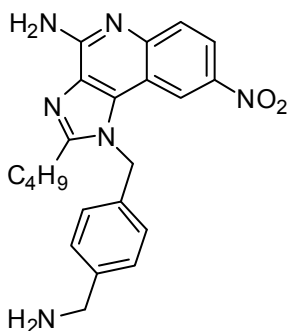
**Synthesis of Compound 11: *tert*-butyl 4-((4-amino-2-butyl-8-nitro-1*H*-imidazo[4,5-*c*]quinolin-1-**



**yl)methyl)benzylcarbamate.**

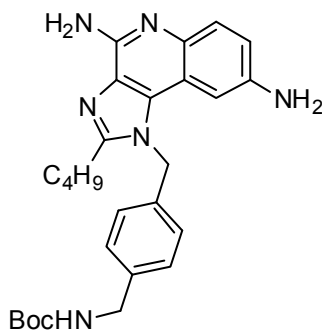
To a solution of **9** (500 mg, 1.16 mmol) in  $H_2SO_4$ , was added  $HNO_3$  (95 mg, 1.511 mmol). The reaction mixture was stirred for 12 hours, followed by neutralization of sulfuric acid by slow addition of sodium carbonate solution. EtOAc was added to this solution to extract the compound, followed by washing with water/brine. The EtOAc fraction was then dried using sodium sulfate and evaporated under vacuum to obtain the residue. The residue as dissolved in MeOH and di-*tert*-butyl dicarbonate was added to it. The reaction was stirred for 30 minutes followed by removal of the solvent under vacuum to obtain the residue, which was purified using column chromatography (7% MeOH/dichloromethane) to obtain the compound **11** (200 mg, 34%).  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.67 (d,  $J$  = 2.5 Hz, 1H), 8.24 – 8.18 (m, 1H), 7.76 (d,  $J$  = 9.2 Hz, 1H), 7.28 (d,  $J$  = 7.0 Hz, 2H), 7.08 (d,  $J$  = 8.1 Hz, 2H), 5.95 (s, 2H), 5.76 (s, 2H), 4.87 (s, 1H), 4.29 (d,  $J$  = 5.5 Hz, 2H), 3.05 – 2.95 (m, 2H), 1.88 (dt,  $J$  = 15.5, 7.6 Hz, 2H), 1.51 (dd,  $J$  = 14.9, 7.3 Hz, 2H), 1.45 (s, 9H), 0.99 (t,  $J$  = 7.4 Hz, 3H).  $^{13}C$  NMR (101 MHz,  $CDCl_3$ )  $\delta$  155.18, 153.33, 148.68, 141.64, 139.53, 133.91, 133.29, 128.41, 127.42, 127.25, 125.92, 121.27, 117.28, 113.88, 48.81, 44.09, 30.00, 28.35, 27.20, 22.54, 13.78. MS (ESI) calculated for  $C_{27}H_{32}N_6O_4$ ,  $m/z$  504.2485, found 505.2541 ( $M + H$ )<sup>+</sup>.

**Synthesis of Compound 11a: 1-(4-(aminomethyl)benzyl)-2-butyl-8-nitro-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



Compound **11** (10 mg, 0.02 mmol) was dissolved in 1 mL solution of HCl/dioxane and stirred for 12 hours. The solvent was then removed under vacuum and the residue was washed with diethyl ether to afford the compound **11a** in quantitative yields.  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  8.77 (d,  $J$  = 1.7 Hz, 1H), 8.47 – 8.41 (m, 1H), 7.98 (d,  $J$  = 9.1 Hz, 1H), 7.54 (d,  $J$  = 7.7 Hz, 2H), 7.30 (d,  $J$  = 7.7 Hz, 2H), 6.09 (s, 2H), 4.11 (s, 2H), 3.12 (t,  $J$  = 7.5 Hz, 2H), 1.98 – 1.88 (m, 2H), 1.59 – 1.49 (m, 2H), 1.00 (t,  $J$  = 7.3 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  158.58, 150.14, 143.93, 137.46, 135.58, 135.36, 133.41, 129.86, 126.29, 125.89, 123.43, 119.40, 117.94, 112.44, 48.52, 42.36, 29.05, 26.44, 21.94, 12.71. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{24}\text{N}_6\text{O}_2$ ,  $m/z$  404.1961, found 405.1993 ( $M + \text{H}$ ) $^+$ .

**Synthesis of Compound 12: *tert*-butyl 4-((4,8-diamino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzylcarbamate.**

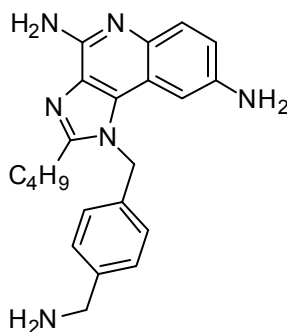


To a solution of **11** (190 mg, 0.377 mmol) in anhydrous MeOH, were added a catalytic amount of Pd/C and the reaction mixture was subjected to hydrogenation at 60 psi hydrogen pressure for 4 hours. The reaction mixture was then filtered through celite and the filtrate was evaporated under



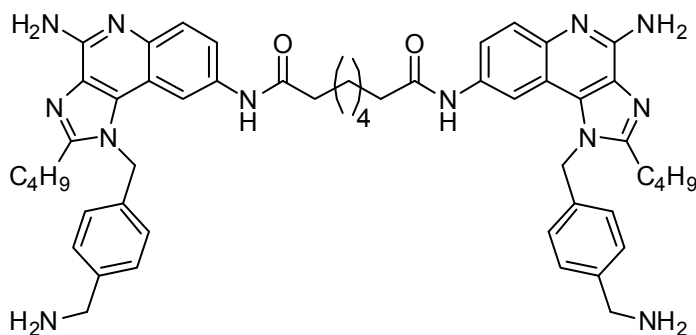
vacuum to obtain the compound **12** (160 mg, 90%).  $^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  7.49 (d,  $J$  = 8.8 Hz, 1H), 7.25 (d,  $J$  = 8.1 Hz, 2H), 7.14 (d,  $J$  = 2.3 Hz, 1H), 7.02 (d,  $J$  = 8.1 Hz, 2H), 6.97 (dd,  $J$  = 8.9, 2.4 Hz, 1H), 5.75 (s, 2H), 4.19 (s, 2H), 2.92 – 2.86 (m, 2H), 1.73 (dt,  $J$  = 15.4, 7.6 Hz, 2H), 1.43 (s, 9H), 0.92 (t,  $J$  = 7.4 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  154.56, 148.78, 142.89, 139.42, 136.71, 134.64, 133.60, 127.53, 125.80, 125.57, 118.16, 115.16, 103.42, 78.81, 43.18, 29.41, 27.33, 26.41, 22.01, 12.67. MS (ESI) calculated for  $\text{C}_{27}\text{H}_{34}\text{N}_6\text{O}_2$ ,  $m/z$  474.2743, found 475.2733 ( $\text{M} + \text{H}$ ) $^+$ .

**Synthesis of Compound 12a: 1-(4-(aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinoline-4,8-diamine.**



Compound **12** (10 mg, 0.021 mmol) was dissolved in 1 mL of HCl/dioxane solution and stirred for 12 hours. The solvent was then removed under vacuum and the residue was washed with diethyl ether to obtain the compound **12a** in quantitative yields.  $^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.10 (s, 1H), 7.97 (d,  $J$  = 8.9 Hz, 1H), 7.67 (dd,  $J$  = 8.9, 2.2 Hz, 1H), 7.51 (d,  $J$  = 8.2 Hz, 2H), 7.23 (d,  $J$  = 8.1 Hz, 2H), 6.04 (s, 2H), 4.12 (s, 2H), 3.03 (t,  $J$  = 7.6 Hz, 2H), 1.92 – 1.84 (m, 2H), 1.53 – 1.43 (m, 2H), 0.96 (t,  $J$  = 7.4 Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz, DMSO)  $\delta$  156.84, 147.83, 135.56, 134.67, 133.58, 129.58, 129.54, 126.08, 125.63, 124.97, 119.52, 113.08, 48.08, 41.64, 29.09, 26.17, 21.75, 13.66. MS (ESI) calculated for  $\text{C}_{22}\text{H}_{26}\text{N}_6$ ,  $m/z$  374.2219, found 375.2508 ( $\text{M} + \text{H}$ ) $^+$ .

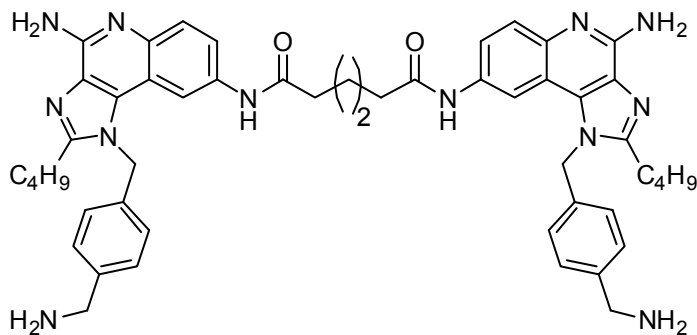
**Synthesis of Compound 13b: *N*<sup>1</sup>,*N*<sup>6</sup>-bis(4-amino-1-(4-(aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-8-yl)octanediamide.**



To a solution of **12** (74 mg, 0.156 mmol) in anhydrous THF, were added triethylamine (39 mg, 0.39 mmol) and suberoyl chloride (15 mg, 0.07 mmol), and the reaction mixture was stirred for 1 hour. The solvent was then removed under vacuum and the residue was purified using column chromatography (20% MeOH/dichloromethane) to obtain the *bis-N*-Boc protected compound which was then dissolved in 1 mL of HCl/dioxane solution and stirred for 14 hours. The solvent was then removed under vacuum and the residue was washed with diethyl ether to afford the compound **13b** (12 mg, 19%). <sup>1</sup>H NMR (500 MHz, MeOD)  $\delta$  8.62 (s, 2H), 7.70 – 7.65 (m, 2H), 7.61 (d, *J* = 9.0 Hz, 2H), 7.44 (d, *J* = 7.9 Hz, 4H), 7.18 (d, *J* = 7.7 Hz, 4H), 5.93 (s, 4H), 4.07 (s, 4H), 2.98 (t, *J* = 7.5 Hz, 4H), 2.37 (dd, *J* = 16.6, 9.4 Hz, 4H), 1.85 (dt, *J* = 15.0, 7.6 Hz, 4H), 1.71 (s, 4H), 1.52 – 1.40 (m, 8H), 0.94 (t, *J* = 7.3 Hz, 6H). <sup>13</sup>C NMR (126 MHz, MeOD)  $\delta$  174.75, 159.05, 149.75, 137.64, 137.34, 137.11, 134.51, 131.24, 130.93, 127.87, 126.22, 123.18, 119.97, 114.28, 111.99, 49.82, 43.82, 37.93, 30.27, 30.01, 27.81, 26.63, 23.36, 14.11. MS (ESI) calculated for C<sub>52</sub>H<sub>62</sub>N<sub>12</sub>O<sub>2</sub>, *m/z* 886.5119, found 909.5031 (*M* + Na<sup>+</sup>) and 444.2632 (*M* + 2H)<sup>2+</sup>.

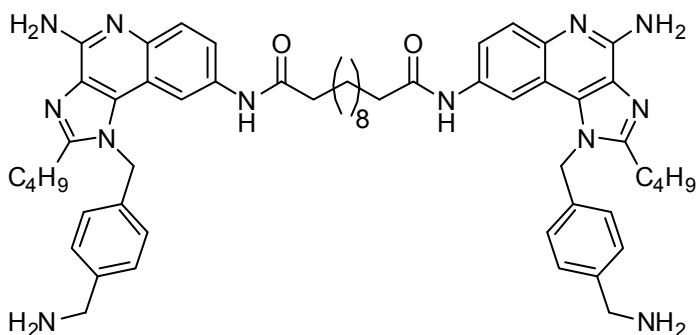
**Compounds 13a and 13c were synthesized similarly as described for compound 13b.**

**Synthesis of Compound 13a: *N*<sup>1</sup>,*N*<sup>6</sup>-bis(4-amino-1-(4-(aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-8-yl)adipamide.**



$^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.57 (s, 2H), 7.62 – 7.54 (m, 4H), 7.35 (d,  $J$  = 8.1 Hz, 4H), 7.10 (d,  $J$  = 8.0 Hz, 4H), 5.85 (s, 4H), 3.98 (s, 4H), 2.90 (t,  $J$  = 7.6 Hz, 4H), 2.36 (s, 4H), 1.77 (dt,  $J$  = 15.2, 7.6 Hz, 4H), 1.73 – 1.61 (m, 4H), 1.45 – 1.30 (m, 4H), 0.86 (t,  $J$  = 7.4 Hz, 6H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  174.43, 159.11, 149.83, 137.66, 137.40, 137.16, 134.53, 131.33, 130.94, 127.89, 126.30, 123.18, 120.01, 114.38, 112.05, 49.82, 43.83, 37.65, 30.32, 27.81, 26.28, 23.37, 14.12. MS (ESI) calculated for  $\text{C}_{50}\text{H}_{58}\text{N}_{12}\text{O}_2$ ,  $m/z$  858.4806, found 859.4131 ( $\text{M} + \text{H}$ ) $^+$  and 430.2113 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

**Synthesis of Compound 13c:  $N',N'^{12}$ -bis(4-amino-1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-8-yl)dodecanediamide.**



$^1\text{H}$  NMR (500 MHz, MeOD)  $\delta$  8.63 (s, 2H), 7.66 (d,  $J$  = 9.0 Hz, 2H), 7.58 (d,  $J$  = 8.9 Hz, 2H), 7.41 (d,  $J$  = 7.9 Hz, 4H), 7.15 (d,  $J$  = 7.8 Hz, 4H), 5.91 (s, 4H), 4.04 (s, 4H), 2.96 (t,  $J$  = 7.5 Hz, 4H), 2.33 (t,  $J$  = 7.1 Hz, 4H), 1.83 (dt,  $J$  = 15.2, 7.7 Hz, 4H), 1.65 (s, 4H), 1.44 (dq,  $J$  = 14.7, 7.3 Hz, 4H), 1.38 – 1.21 (m, 12H), 0.92 (t,  $J$  = 7.3 Hz, 6H).  $^{13}\text{C}$  NMR (126 MHz, MeOD)  $\delta$  174.82, 159.01, 149.63, 137.68, 137.27, 137.10, 134.50, 131.24, 130.93, 129.84, 127.86, 126.00, 123.16, 120.01, 114.24, 111.92, 49.87, 43.82, 43.75, 38.04, 30.45, 30.34, 30.29, 30.23, 27.78, 26.78, 23.35, 14.11. MS (ESI) calculated for  $\text{C}_{56}\text{H}_{70}\text{N}_{12}\text{O}_2$ ,  $m/z$  942.5745, found 943.5746 ( $\text{M} + \text{H}$ ) $^+$  and 472.2987 ( $\text{M} + 2\text{H}$ ) $^{2+}$ .

**TLR3/7/8 Reporter Gene assays (NF- $\kappa$ B induction):** Agonism assays were performed exactly as described in Chapter 4 using appropriate, stably transfected reporter cell-lines. Antagonism assays were done using the following agonists at a constant concentration: TLR3 Poly(I:C) (10 ng/mL); TLR7: gardiquimod (1  $\mu$ g/mL); TLR8: CL075(1  $\mu$ g/mL) mixed with graded concentrations of the test compounds.

**IFN- $\alpha$  induction in human PBMCs:** Aliquots (100  $\mu$ L) of hPBMCs isolated from blood obtained from healthy human donors after informed consent by conventional Ficoll-Hypaque gradient centrifugation were stimulated for 12 h with graded concentrations of test compounds. The supernatant was isolated by centrifugation, diluted 1:20, and IFN- $\alpha$  was assayed in triplicate using a high-sensitivity human IFN- $\alpha$ -specific ELISA kit (PBL Interferon Source, Piscataway, NJ) as described in Chapter 4.

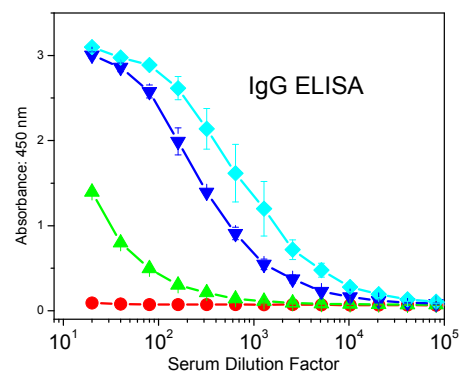
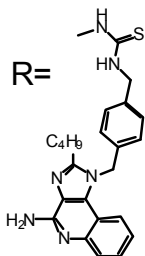
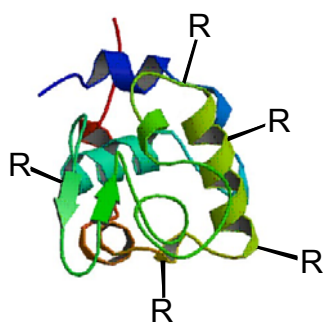
**Transcriptomal profiling in human PBMCs:** Assays were performed as described by us previously.<sup>69</sup> Briefly, peripheral blood mononuclear cells (PBMCs) isolated from fresh, heparin-anticoagulated human blood was stimulated with 20  $\mu$ g/mL of the compounds for two hours, and total RNA was extracted from treated and negative control PBMC samples with QIAamp RNA Blood Mini Kit (Qiagen). 4  $\mu$ g of each of the RNA samples was used for transcriptomal profiling, employing the Human Genome GeneChip U133 plus 2.0 oligonucleotide array (Affymetrix, Santa Clara, CA). Established standard protocols at the KU Genomics Facility were performed on cRNA target preparation, array hybridization, washing, staining and image scanning. The microarray data were collected using the Affymetrix GeneChip Command Console Software (AGCC) and then subjected to quality assessment before further analyses. QC criteria included low background, low noise, detection of positive controls, and a 5'/3' ratio of < 3.0. To facilitate direct comparison of gene expression data between different samples, the GeneChip data were first subjected to preprocessing, which included scaling (in GCOS) data from all chips to a target intensity value of 500 (in Affymetrix Expression Console Software), and further normalization in GeneSpring GX (Agilent

Technologies, Santa Clara, CA). Prior to identifying target genes, genes that were detected as non-expressed in all samples, i.e., those with absence (A) cells were filtered out. Genes whose expression was changed by the test compounds by at least 2-fold (compared to the negative control) were identified to be differentially expressed.

## Chapter 7.

# SELF-ADJUVANTING MODEL PEPTIDE AND PROTEIN ANTIGENS WITH COVALENTLY BOUND TLR-7 AGONISTIC IMIDAZOQUINOLINES

Self-adjuvanting vaccine



## 7.1. Introduction.

One aspect of our work in the area of evaluating TLR agonists as vaccine adjuvants<sup>69;137;150</sup> focuses on developing self-adjuvanting vaccine constructs, i.e., antigen covalently coupled to a suitable adjuvant. The premise of covalently decorating protein antigens with potential adjuvants offers the possibility of drastically reducing systemic exposure of the adjuvant, and yet maintaining relatively high local concentrations at the site of vaccination.<sup>214</sup> Most self-adjuvanting vaccine constructs to date have utilized TLR-2 agonistic 2,3-*bis*-(palmitoyloxy)propyl-cysteinyl peptides as the adjuvant.<sup>215-220</sup> The conjugation of the poorly soluble lipopeptide adjuvant to antigen has limited this approach to peptide<sup>216-219;221</sup> or glycopeptide<sup>220</sup> antigens, since native proteins are often irrevocably denatured under the coupling conditions employed, with potential loss of key epitopes. These limitations have recently been addressed by appending to the lipopeptide a long, water-solubilizing poly-lysine or polyethylene glycol moiety, and terminating in a free thiol.<sup>222</sup> However, in addition to the potential problem of oxidation of lipopeptide thiol to the disulfide, free exposed thiols in proteins are rare.<sup>223</sup> Furthermore, TLR2 ligation has been associated with Th2 and Th17 responses<sup>224;225</sup> which may, in many instances, be undesirable.

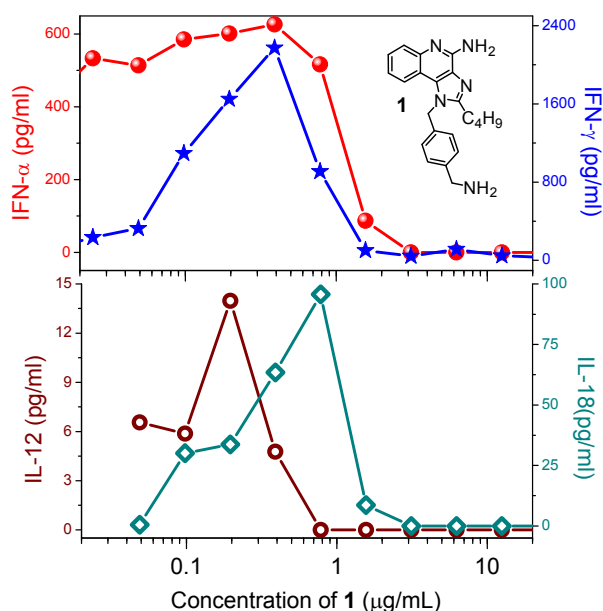
As noted in Chapters 1 and 2, we have identified TLR7 agonists to be extraordinarily potent, stimulating virtually all subsets of lymphocytes, and yet without inducing dominant proinflammatory cytokine responses.<sup>69</sup> A TLR7/8 dual-agonistic *N*<sup>1</sup>-(4-aminomethyl)benzyl substituted imidazoquinoline **1** (described as **5d** in Chapter 5) served as a convenient precursor for the syntheses of isothiocyanate and maleimide derivatives for covalent attachment to free amine and thiol groups of peptides and proteins. **1** was also amenable to direct reductive amination with maltoheptaose without significant loss of activity. Covalent conjugation of the isothiocyanate derivative **2** to  $\alpha$ -lactalbumin could be achieved under mild, non-denaturing conditions, in a controlled

manner and with full preservation of antigenicity. The self-adjuvanting  $\alpha$ -lactalbumin construct induced robust, high-affinity immunoglobulin titers in murine models. The premise of covalently decorating protein antigens with adjuvants offers the possibility of drastically reducing systemic exposure of the adjuvant, and yet eliciting strong, Th1-biased immune responses.

## 7.2. Results and Discussion.

Desirous of specifically identifying chemotypes with strong Th1-biased immunostimulatory signatures, we implemented additional screens examining the induction of Type I<sup>226-228</sup> and Type II<sup>229;230</sup> interferons (IFN- $\alpha/\beta$  and IFN- $\gamma$ , respectively), Interleukin-12 (IL-12),<sup>231;232</sup> and Interleukin-18 (IL-18) using human PBMCs,<sup>233-235</sup> all of which are strongly associated with dominant Th1 outcomes. These experiments were most instructive in that they enabled us to determine that of all of the diverse chemotypes of our rapidly expanding libraries of TLR agonists,<sup>132;137;150;236</sup> an *N*<sup>1</sup>-(4-aminomethyl)benzyl substituted imidazoquinoline **1** displayed a prominent Th1 bias (**Fig. 1**).

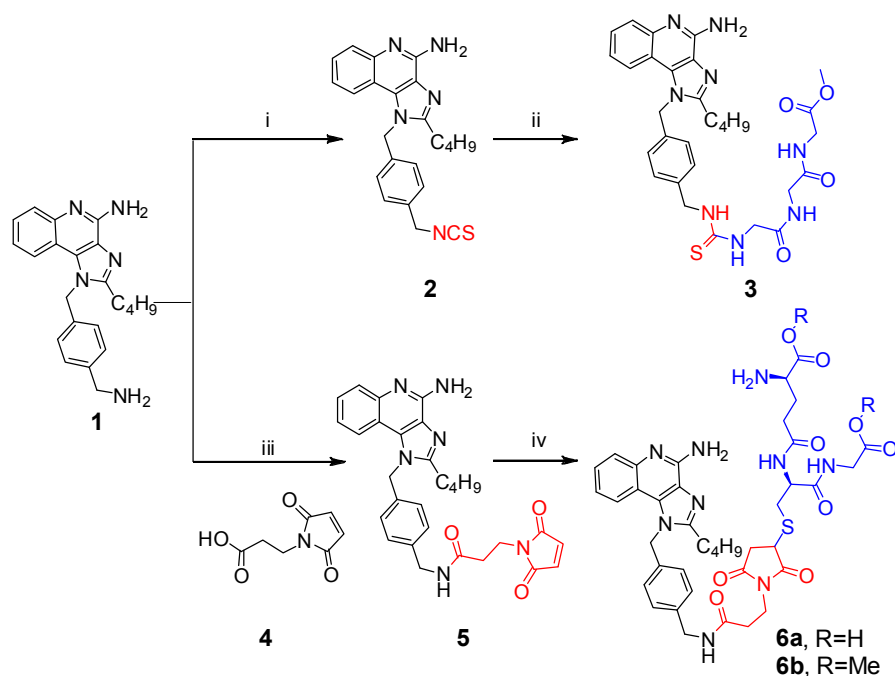
**Fig. 1.** Induction of IFN- $\alpha$ , IFN- $\gamma$  (top) and IL-12 and IL-18 (bottom) by **1** in human PBMCs. IFN and cytokine levels were quantified by ELISA. Results of a representative experiment are shown.





Because the aqueous solubility of **1** and several of its congeners were excellent, we elected to evaluate direct covalent coupling to free amines and thiols on protein antigens via the introduction of conventional isothiocyanate and maleimide electrophilic handles on the imidazoquinoline scaffold. Our earlier attempts at incorporating the electrophilic isothiocyanate functionality on the C2-sidechain for covalent adduction to peptides and proteins had met with failure in that such adducts were bereft of TLR7-agonistic activity,<sup>132</sup> and it occurred to us that not only could the free primary amine on the *N*<sup>1</sup> substituent of **1** be converted directly to the corresponding isothiocyanate (**2**, Scheme 1), but also an additional, thiol-specific maleimide electrophile could be attached via a spacer group (**5**, Scheme 1). The isothiocyanate derivative **2** reacted well with a tri-glycine methyl ester model peptide, yielding the adduct **3** (Scheme 1), which was purified to homogeneity, and found to be active (**Fig. 2**). Facile adduction of the maleimide derivative **5** with glutathione (reduced) also afforded the **6a** in near-quantitative yields.

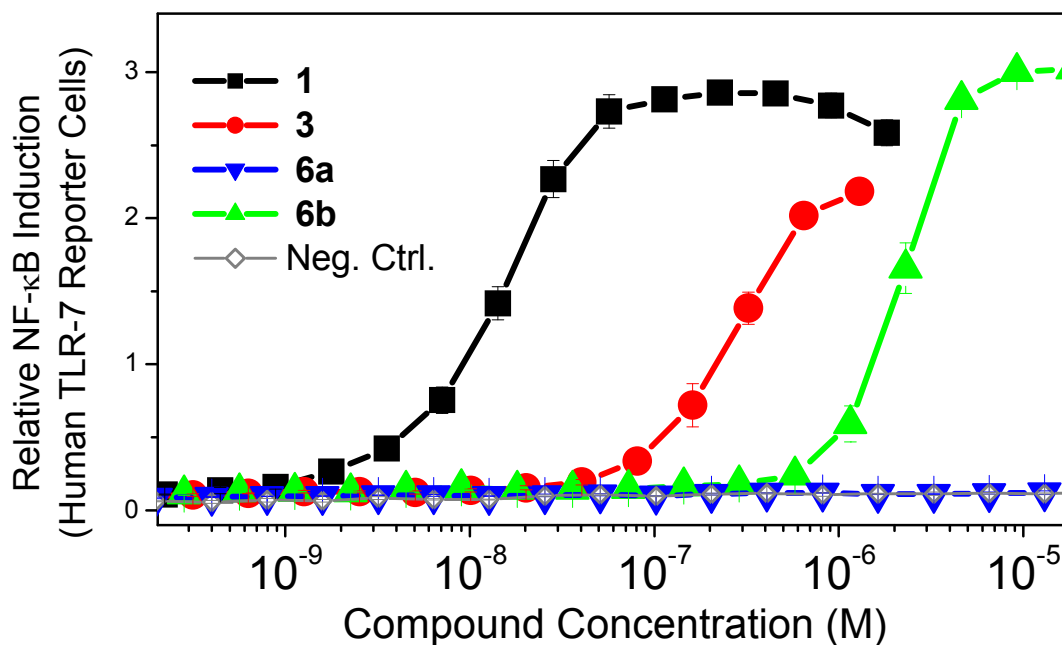
**Scheme 1.** Syntheses of isothiocyanate and maleimide derivatives of **1**, and their corresponding model peptide conjugates.



Reagents: i. CS<sub>2</sub>, Et<sub>3</sub>N, DMAP, (Boc)<sub>2</sub>O, CH<sub>2</sub>Cl<sub>2</sub>; ii. Tri-glycine methyl ester, Et<sub>3</sub>N, MeOH; iii. HBTU, Et<sub>3</sub>N, DMAP, DMF; iv. glutathione-reduced (GSH) or GSH dimethyl ester, Et<sub>3</sub>N, MeOH/CH<sub>2</sub>Cl<sub>2</sub>.

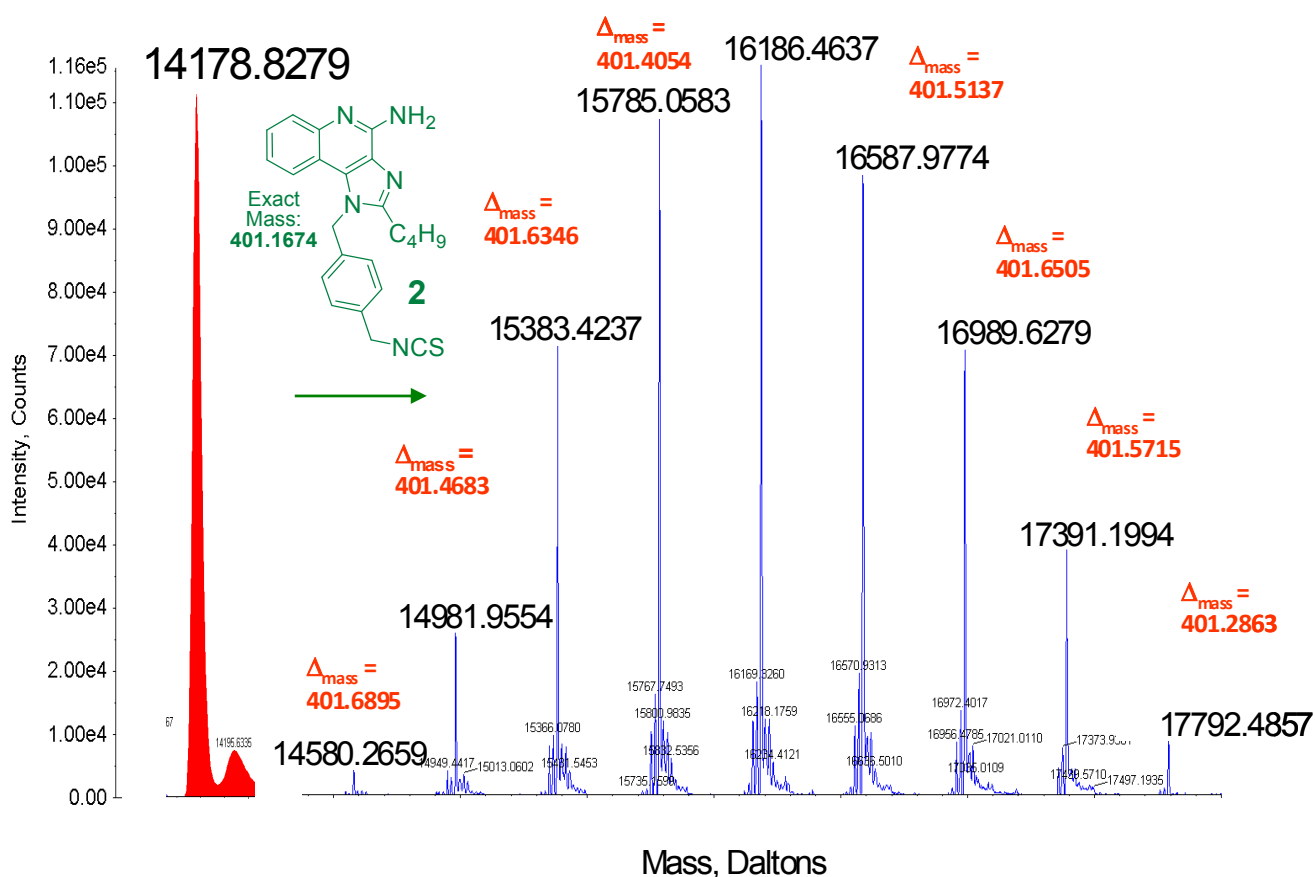
The imidazoquinolines themselves are small, non-polar, and basic, and therefore gain access to the endolysosomal compartment in which TLR7 is predominantly sequestered. The human embryonic kidney reporter cell lines stably transfected with TLR7 (and reporter secreted alkaline phosphatase genes) that we employ in our primary screen are not professional phagocytic cells, and we were concerned if the trans-membrane permeability of the bulky, dianionic adduct **6a** would be sufficient to trigger activation, and we therefore also obtained the conjugate of **5** with the dimethyl ester of reduced glutathione (**6b**). As could be predicted, the adducts **3** and **6b** retained activity ( $EC_{50}$ : 269 nM and 2.2  $\mu$ M), while **6a** was completely inactive (**Fig. 2**), indicating that trans-cellular transport of the polar adduct with two net negative charges was insufficient.

**Fig. 2.** TLR7-agonistic activities of imidazoquinoline analogues in a human TLR7-specific reporter gene assay.



We elected to work with bovine  $\alpha$ -lactalbumin as a model antigen for self-adjuvanting vaccine constructs not only because it lent itself eminently well to rigorous characterization by electrospray ionization mass spectrometry (ESI-MS) methods (**Fig. 3**), but also because it is being evaluated as a potential antigen for breast cancer vaccines.<sup>237</sup>

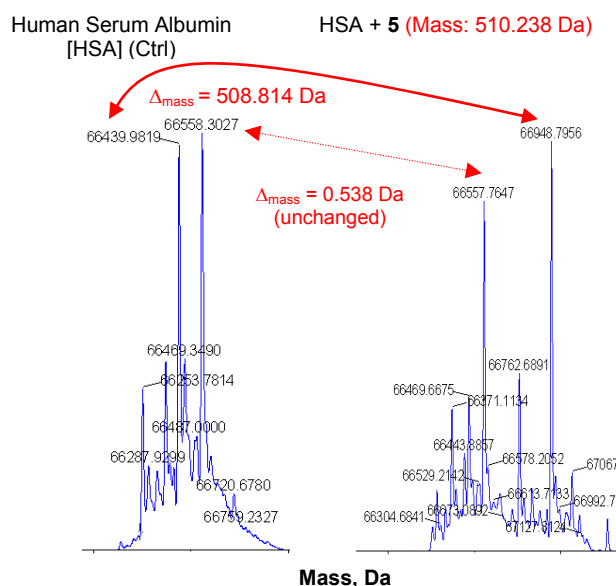
**Fig. 3.** Deconvoluted positive-mode ESI-MS spectra of native bovine  $\alpha$ -lactalbumin (left) showing a mass of 14178.83 Da, and  $\alpha$ -lactalbumin reacted with 5 eq. of **2**, resulting in a stochastic coupling of the adjuvant with the centroid of the mass distribution corresponding to exactly 5 units of imidazoquinoline per protein molecule.



We reacted 5 equivalents of **2** with bovine  $\alpha$ -lactalbumin in isotonic aqueous buffer at pH 8.5. Direct LC-ESI-MS evidence was obtained for covalent adduction of **2** with the protein, indicating a remarkably beautiful and precise Gaussian distribution of adducted species, with the preponderant conjugate corresponding to a 1:5 molar ratio of protein:**2** (**Fig. 3**).

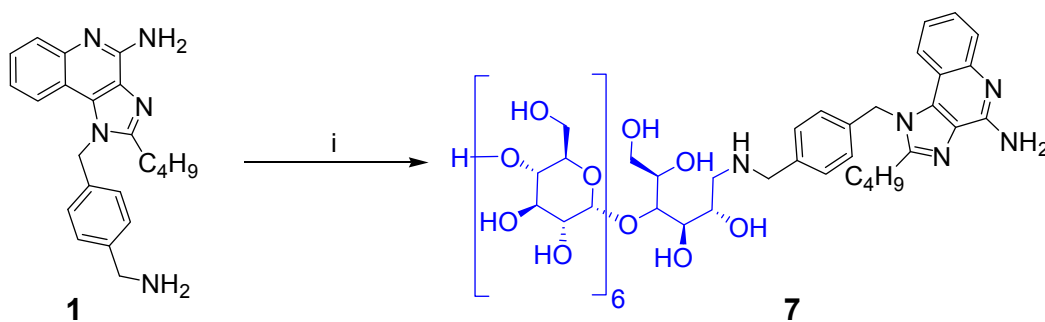
Encouraged by these results, we also attempted to conjugate **5** with human serum albumin (HSA), a 66 kDa protein with a single free thiol. We elected to use clinical grade (meant for human parenteral use, formulated with amino acids) HSA rather than the purer, ‘essentially fatty acid free’ protein available commercially. Furthermore, HSA is a carrier protein which binds promiscuously to a vast range of ligands including heavy metals, bilirubin, fatty acids, etc. We anticipated for these reasons, that deconvoluted direct electrospray-time-of-flight mass (ESI-TOF) spectra would be polydisperse and microheterogeneous. In the HSA-alone control sample, we found two major peaks corresponding to 66439.9819 and 66558.3027 Da (**Fig. 4**). Reaction of HSA with **5** produced a shift in one of the peaks with a  $\Delta_{\text{mass}}$  of 508.814 Da which corresponds to the maleimide derivative **5** within instrument error (1.424 Da at 66KDa = 21.2 parts per million). The other peak at 66558 Da had remained unaltered upon addition of excess thiol-specific maleimide analogue **5**, suggesting that the thiol was unreactive (**Fig. 4**). An examination of the difference between the species with the free, reactive thiol (66439 Da) and the unreactive thiol (66558 Da) in the control sample suggests that the ‘blocking’ group is cysteine (expected exact mass of cysteine – 1 proton [disulfide] = 118.02; observed  $\Delta_{\text{mass}}$  = 118.321 Da).

**Fig. 4.** Covalent coupling of the thiol-specific maleimide derivative **5** with human serum albumin showing addition of a single equivalent of **5** to albumin, as examined by LC-ESI-TOF. An excess (5 equiv.) of **5** was used.



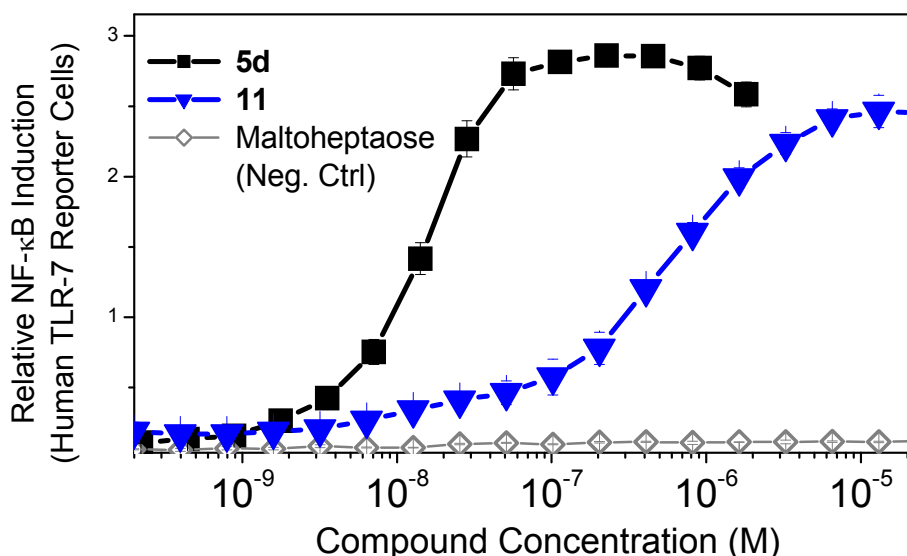
Aside from engineering otherwise feebly immunogenic peptide and subunit protein vaccines for the induction of strong CTL responses, we are also interested in polysaccharide vaccines which have proved enormously useful in the prevention of infections by bacterial pathogens such as *N. meningitidis* and *H. influenzae*.<sup>238-240</sup> Bacterial polysaccharides, unlike conventional protein antigens, have been considered classic T cell-independent antigens that do not elicit cell-mediated immune responses but rather elicit non-anamnestic responses characterized by low-affinity IgM and restricted classes of IgG immunoglobulins without the recruitment of T cell help. Conversion to canonical, T lymphocyte-dependent responses require their covalent coupling to immunogenic ‘carrier proteins’ such as diphtheria toxoid.<sup>241;242</sup> This appears not to be the case for zwitterionic polysaccharides, however, which elicit potent CD4<sup>+</sup> T cell responses.<sup>243;244</sup> We are keen to re-examine the structural determinants of T-dependent and -independent humoral responses, especially in light of recent findings of intrinsic TLR2 activation by zwitterionic polysaccharides,<sup>245;246</sup> and we are currently testing the hypothesis that polysaccharides with covalently linked TLR stimuli may be superior to carrier proteins. The free primary amine on the *N*<sup>1</sup> substituent of **1** lent itself eminently well to direct reductive amination with maltoheptaose, a model oligosaccharide with a reducing terminal maltose unit (**7**, Scheme 2) which, gratifyingly, was found to be active in TLR7 assays (EC<sub>50</sub>: 528 nM; **Fig. 5**). We are currently exploring conjugating *N. meningitidis* Group C polysaccharide<sup>247</sup> with **1**.

**Scheme 2.** Syntheses of the maltoheptaose conjugate.



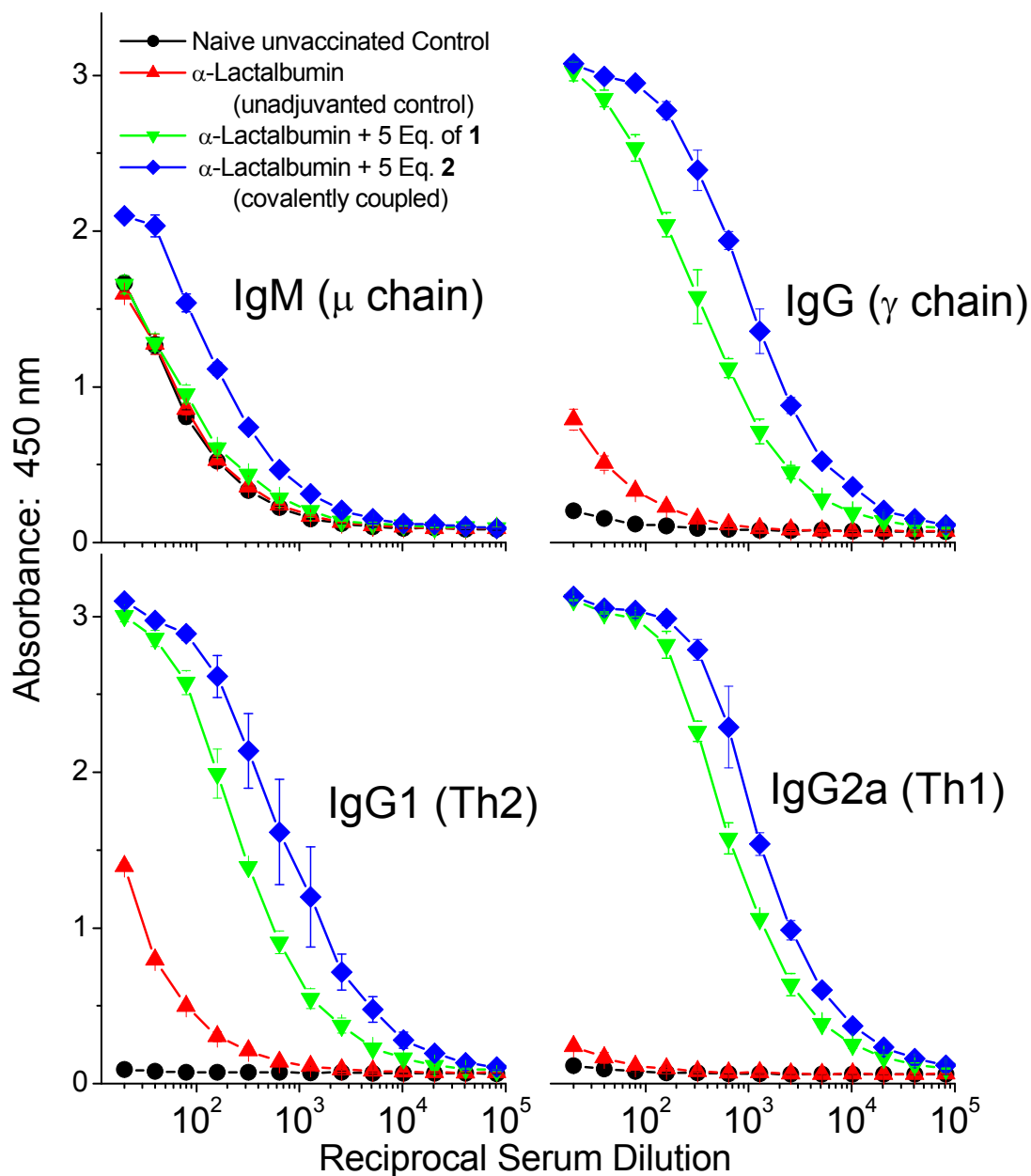
Reagents: i. Maltoheptaose, CH<sub>3</sub>CO<sub>2</sub>H, Macroporous resin-bound CNBH<sub>3</sub>, 50 °C, DMF.

**Fig. 5.** TLR7-agonistic activities of imidazoquinoline-maltoheptaose conjugate in a human TLR7-specific reporter gene assay.



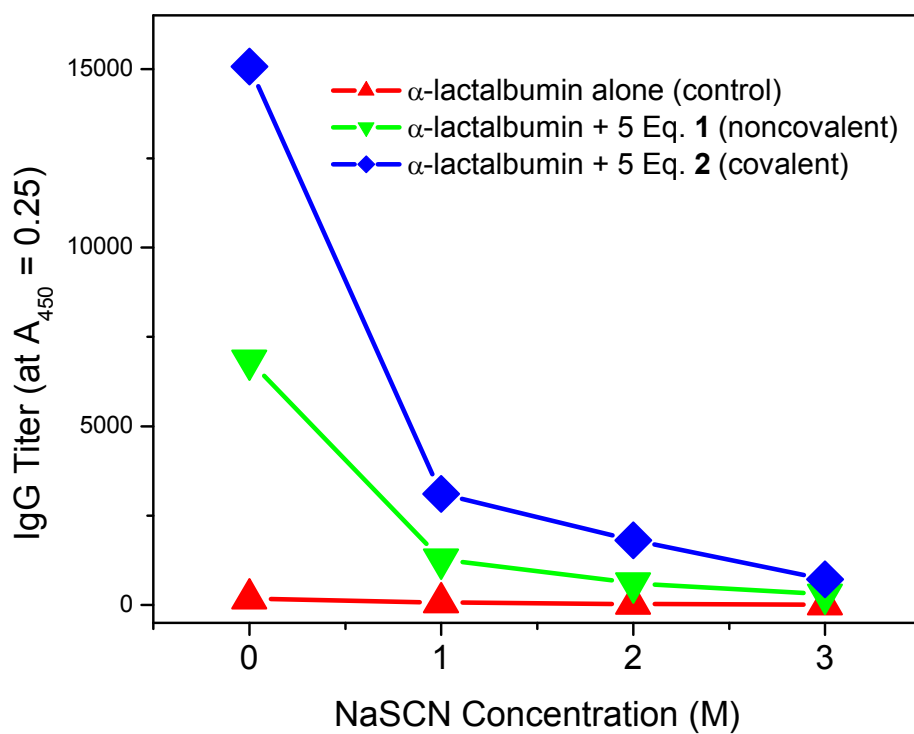
It was of particular interest to us to evaluate the well-characterized **2**: $\alpha$ -lactalbumin conjugate (**Fig. 3**) as a test-case for self-adjuvanting subunit vaccine construct. We specifically asked whether the conjugation procedure would preserve antigenicity of the protein, and whether the covalently-adducted construct would be superior to a physical mixture of  $\alpha$ -lactalbumin and **1**. Cohorts (5 per group) of outbred CF-1 mice were immunized with 50  $\mu$ g per animal of  $\alpha$ -lactalbumin, or 50  $\mu$ g of  $\alpha$ -lactalbumin covalently conjugated with 5 equivalents of **2**, or a mixture of 50  $\mu$ g of  $\alpha$ -lactalbumin and 5 equivalents of **1**. The animals were boosted once after two weeks following the priming dose, and bled after an additional week.  $\alpha$ -lactalbumin-specific IgM, IgG, as well as IgG1 and IgG2a (isotypes characteristic of Th2 and Th1 responses,<sup>248</sup> respectively) were quantified by ELISA. As shown in **Fig. 6**, dramatic enhancements in antibody titers were observed with both covalently- and non-covalently adjuvanted protein (relative to  $\alpha$ -lactalbumin alone); we also observed modest, but consistent, and statistically significant differences in titers between the covalently coupled self-adjuvanting construct, and mixture of antigen and adjuvant, indicating that self-adjuvanting subunit protein vaccines may indeed be generated with full preservation of antigenicity.

**Fig. 6.** Immunoglobulin profiles in outbred CF-1 mice immunized on Day 0 with 50  $\mu\text{g}/\text{animal}$  of  $\alpha$ -lactalbumin, or  $\alpha$ -lactalbumin covalently coupled with 5 equivalents of **2**, or  $\alpha$ -lactalbumin mixed with 5 equivalents of **1**. Animals (5 per cohort) were boosted once on Day 14 exactly as mentioned above, and bled on Day 21.  $\alpha$ -lactalbumin-specific immunoglobulin levels were quantified by standard antibody-capture ELISA, performed in liquid handler-assisted 384-well format.



Examination of the affinity of antigen-specific IgG using conventional chaotropic ELISA<sup>249;250</sup> also indicates higher quality IgG (**Fig. 7**) elicited by the self-adjuvanting construct.

**Fig. 7.** Affinity IgG ELISA showing antibody titer as a function of chaotrope (NaSCN) concentration. IgG titers on the ordinate axis were calculated from absorbance values at 0.25 (which corresponds to  $3\sigma$  above that of naïve controls).





### 7.3. Conclusions.

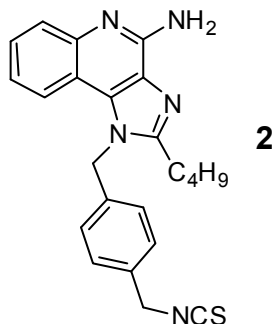
In conclusion, our continuing exploration of the TLR7-agonistic imidazoquinoline chemotype in tandem with expanded secondary and tertiary screens designed to specifically evaluate Th1-orienting immunostimulatory profiles have enabled the identification of **1**, whose free amine group can be conveniently exploited in constructing covalent conjugates with peptides, proteins, as well as polysaccharides with preservation of immunostimulatory activity.

The feasibility of covalently coupling a small-molecule TLR7 agonist to proteins under mild, non-denaturing conditions to yield self-adjuvanting subunit vaccines is evident, and may have considerable practical value in significantly reducing systemic exposure of the adjuvant, and yet inducing high innate and adaptive immune responses. We are currently examining CD8<sup>+</sup> CTL responses elicited by such constructs.

### 7.4. Experimental.

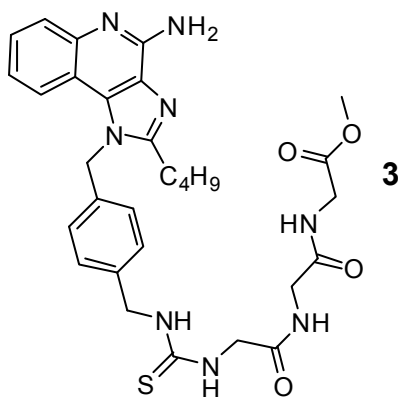
All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried (120 °C) glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using RediSep Rf 'Gold' high performance silica columns on CombiFlash Rf instruments unless otherwise mentioned, while thin-layer chromatography was carried out on silica gel CCM pre-coated aluminum sheets. Purity for all final compounds was confirmed to be greater than 97% by LC-MS using a Zorbax Eclipse Plus 4.6 mm x 150 mm, 5 µm analytical reverse phase C<sub>18</sub> column with H<sub>2</sub>O-isopropanol or H<sub>2</sub>O-CH<sub>3</sub>CN gradients and an Agilent ESI-TOF mass spectrometer (mass accuracy of 3 ppm) operating in the positive ion acquisition mode. All the compounds synthesized were obtained as solids.

**Synthesis of Compound 2: 2-Butyl-1-(4-(isothiocyanatomethyl)benzyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine.**



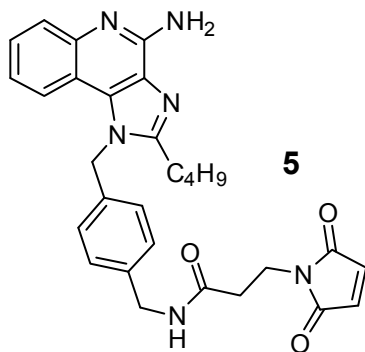
To a solution of **1** (150 mg, 0.35 mmol) in anhydrous dichloromethane, were added carbon disulfide (266 mg, 3.5 mmol) and triethylamine (106 mg, 1.05 mmol). The reaction mixture was stirred for an hour and then was cooled to 0 °C. Di-*tert*-butyl dicarbonate (76 mg, 0.35 mmol) and a catalytic amount of DMAP were added to the reaction mixture. The reaction mixture was stirred for 18 hours and then the solvent was removed under vacuum. The residue was purified using column chromatography (7% MeOH/ dichloromethane) to obtain the compound **2** (105 mg, 75%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.87 – 7.83 (m, 1H), 7.68 (dd, *J* = 8.3, 0.8 Hz, 1H), 7.51 – 7.45 (m, 1H), 7.32 (d, *J* = 8.2 Hz, 2H), 7.23 – 7.17 (m, 1H), 7.10 (d, *J* = 8.2 Hz, 2H), 6.52 (s, 2H), 5.78 (s, 2H), 4.71 (s, 2H), 2.94 – 2.86 (m, 2H), 1.82 (dt, *J* = 15.5, 7.6 Hz, 2H), 1.52 – 1.41 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 155.19, 150.27, 135.10, 134.65, 134.57, 128.20, 127.95, 126.10, 125.92, 124.08, 123.59, 119.94, 113.99, 48.74, 48.21, 29.71, 27.12, 22.47, 13.76. MS (ESI) calculated for C<sub>23</sub>H<sub>23</sub>N<sub>5</sub>S, *m/z* 401.17, found 402.18 (*M* + *H*)<sup>+</sup>.

**Synthesis of Compound 3: Methyl 1-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)phenyl)-6,9-dioxo-3-thioxo-2,4,7,10-tetraazadodecan-12-oate.**



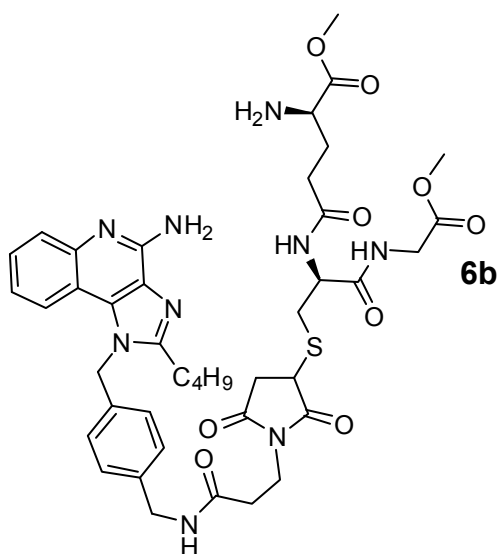
To a solution of **2** (15 mg, 0.037 mmol) in anhydrous MeOH, were added triethylamine (6 mg, 0.056 mmol) and methyl 2-(2-(2-aminoacetamido)acetamido)acetate hydrochloride (11mg, 0.044 mmol). The reaction was heated at 45 °C for 4 hours. The solvent was then removed under vacuum and the residue was purified using column chromatography (14 % MeOH/dichloromethane) to obtain the compound **3** (5 mg, 22%). <sup>1</sup>H NMR (400 MHz, DMSO) δ 8.36 – 8.14 (m, 3H), 7.79 (d, *J* = 7.7 Hz, 1H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.33 (dd, *J* = 11.2, 4.1 Hz, 1H), 7.25 (d, *J* = 8.2 Hz, 2H), 7.05 (t, *J* = 7.1 Hz, 1H), 7.00 (d, *J* = 8.1 Hz, 2H), 6.59 (s, 2H), 5.85 (s, 2H), 4.61 (s, 2H), 4.11 (s, 2H), 3.83 (d, *J* = 5.9 Hz, 2H), 3.75 (d, *J* = 5.9 Hz, 2H), 3.62 (s, 3H), 2.96 – 2.86 (m, 2H), 1.72 (dt, *J* = 15.3, 7.6 Hz, 2H), 1.45 – 1.31 (m, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). MS (ESI) calculated for C<sub>30</sub>H<sub>36</sub>N<sub>8</sub>O<sub>4</sub>S, *m/z* 604.26, found 605.27 (*M* + H)<sup>+</sup>.

**Synthesis of Compound 5:** *N*-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzyl)-3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanamide.



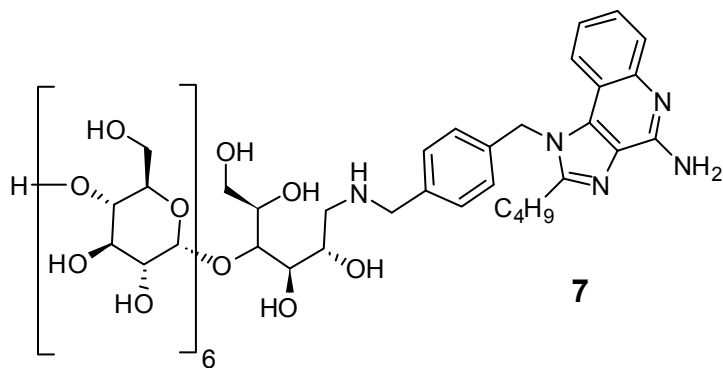
To a solution of **4** (30 mg, 0.18 mmol) in anhydrous DMF, were added triethylamine (50 mg, 0.49 mmol), HBTU (68 mg, 0.18 mmol), a catalytic amount of DMAP and **1** (70 mg, 0.16 mmol). The reaction mixture was stirred for 14 hours and then the solvent was removed under vacuum. The residue was dissolved in ethyl acetate and washed with water. The ethyl acetate fraction was then dried using Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum to obtain the residue, which was purified using column chromatography (5% MeOH/dichloromethane) to obtain the compound **5** (65 mg, 80%). <sup>1</sup>H NMR (500 MHz, MeOD) δ 7.97 (dd, *J* = 8.4, 0.7 Hz, 1H), 7.74 – 7.71 (m, 1H), 7.64 (ddd, *J* = 8.4, 7.2, 1.2 Hz, 1H), 7.38 (ddd, *J* = 8.4, 7.2, 1.2 Hz, 1H), 7.26 (d, *J* = 8.3 Hz, 2H), 7.04 (d, *J* = 8.3 Hz, 2H), 6.73 (s, 2H), 5.93 (s, 2H), 4.27 (s, 2H), 3.75 (t, *J* = 7.0 Hz, 2H), 3.02 – 2.97 (m, 2H), 2.47 (t, *J* = 7.0 Hz, 2H), 1.85 (dt, *J* = 21.1, 7.6 Hz, 2H), 1.46 (dq, *J* = 14.8, 7.4 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 172.88, 172.10, 159.09, 150.46, 140.15, 137.66, 135.44, 135.36, 135.21, 130.99, 129.71, 126.83, 126.50, 125.89, 123.02, 119.59, 114.25, 49.85, 43.65, 35.59, 35.40, 30.35, 27.78, 23.33, 14.12. MS (ESI) calculated for C<sub>29</sub>H<sub>30</sub>N<sub>6</sub>O<sub>3</sub>, *m/z* 510.24, found 511.25 (*M* + H)<sup>+</sup>.

**Synthesis of Compound 6b: (2*R*)-methyl 2-amino-5-((2*S*)-3-(1-(3-(4-((4-amino-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)methyl)benzylamino)-3-oxopropyl)-2,5-dioxopyrrolidin-3-ylthio)-1-(2-methoxy-2-oxoethylamino)-1-oxopropan-2-ylamino)-5-oxopentanoate.**



To a solution of **5** (15 mg, 0.03 mmol) in anhydrous MeOH and a few drops of anhydrous dichloromethane, were added triethylamine (8 mg, 0.08 mmol) and glutathione reduced dimethyl ester (20 mg, 0.06 mmol). [Glutathione-reduced dimethyl ester was obtained from glutathione-reduced by stirring in mixture of methanol and 1 mL of HCl/dioxane solution for 30 hours, followed by removal of the solvent under vacuum]. The reaction mixture was stirred for 30 minutes, followed by removal of solvent under vacuum. The residue was then purified using column chromatography (20% MeOH/dichloromethane) to obtain the compound **6b** (5 mg, 60%). <sup>1</sup>H NMR (500 MHz, MeOD) δ 7.75 (d, *J* = 8.3 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.36 (t, *J* = 7.3 Hz, 1H), 7.17 (d, *J* = 8.2 Hz, 2H), 7.06 (t, *J* = 7.7 Hz, 1H), 6.94 (d, *J* = 8.1 Hz, 2H), 5.79 (s, 2H), 4.19 (qd, *J* = 15.2, 6.7 Hz, 2H), 3.84 (s, 2H), 3.68 – 3.61 (m, 5H), 3.58 (s, 3H), 3.54 (dt, *J* = 10.7, 4.5 Hz, 1H), 3.14 – 2.93 (m, 3H), 2.93 – 2.82 (m, 2H), 2.39 (td, *J* = 6.9, 2.4 Hz, 2H), 2.36 – 2.27 (m, 3H), 2.05 – 1.91 (m, 1H), 1.90 – 1.78 (m, 1H), 1.75 – 1.66 (m, 2H), 1.40 – 1.30 (m, 2H), 1.21 (t, *J* = 7.3 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H). MS (ESI) calculated for C<sub>41</sub>H<sub>51</sub>N<sub>9</sub>O<sub>9</sub>S, *m/z* 845.35, found 868.33 (M + Na<sup>+</sup>).

### Synthesis of Compound 7:



To a solution of compound **1** (8 mg, 0.019mmol) in anhydrous DMF, were added 3-4 drops of acetic acid, maltoheptaose (20 mg, 0.018 mmol) and macroporous polystyrene-bound cyanoborohydride (15 mg, 0.033 mmol). The reaction mixture was heated at 50 °C for 24 hours. The solution was filtered to remove the solid resin and the filtrate was evaporated under vacuum to obtain the residue which was purified using C<sub>18</sub> reverse-phase column chromatography (40% MeOH/H<sub>2</sub>O) to obtain the

compound **7** (12 mg, 45%). MS (ESI) calculated for  $C_{64}H_{97}N_5O_{35}$ ,  $m/z$  1495.60, found 1518.59 ( $M + Na^+$ ) and 759.83 ( $M + H + Na$ )<sup>2+</sup>.

**Immunoassays for Interferon (IFN)- $\alpha$ , IFN- $\gamma$ , Interleukin (IL)-12, and IL-18.** Fresh human peripheral blood mononuclear cells (PBMC) were isolated from human blood obtained by venipuncture with informed consent and as per institutional guidelines on Ficoll-Hypaque gradients as described elsewhere.<sup>251</sup> Aliquots of PBMCs ( $10^5$  cells in 100  $\mu$ L/well) were stimulated for 12 h with graded concentrations of test compounds. Supernatants were isolated by centrifugation, diluted 1:20, and were assayed in triplicates using a high-sensitivity analyte-specific ELISA kits (PBL Interferon Source, Piscataway, NJ and R&D Systems, Inc., Minneapolis, MN).

**NF- $\kappa$ B induction in human TLR7-expressing reporter gene assays:** The induction of NF- $\kappa$ B was quantified using HEK-Blue-7 cells as previously described by us.<sup>137;178</sup> HEK293 cells were stably transfected with human TLR7, MD2, and secreted alkaline phosphatase (sAP), and were maintained in HEK-Blue™ Selection medium containing zeocin and normocin. Stable expression of secreted alkaline phosphatase (sAP) under control of NF- $\kappa$ B/AP-1 promoters is inducible by the TLR7 (or TLR8) agonists, and extracellular sAP in the supernatant is proportional to NF- $\kappa$ B induction. HEK-Blue cells were incubated at a density of  $\sim 10^5$  cells/mL in a volume of 80  $\mu$ L/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

**Protein Adduction and Mass Spectrometry Experiments:** Bovine  $\alpha$ -lactalbumin (Sigma-Aldrich Chemical Co., St. Louis, MO, and clinical grade human serum (Talecris Biotherapeutics, Research Triangle Park, NC) were incubated with **2** and **5**, respectively at a molar ratio of 1:5

(protein:imidazoquinoline) in aqueous carbonate buffer at pH 8.0 overnight. The adducted proteins were analyzed by reverse-phase LC-ESI-MS performed on a Shimadzu LC system (LC-10AD binary pumps, SCL-10A diode array detector) using a Zorbax 3.0 mm x 150 mm 3.5  $\mu$ m stable-bond C<sub>18</sub> reverse-phase column with a forty-minute binary gradient (CH<sub>3</sub>CN/water, 0.1% HCOOH) from 5% to 95% of CH<sub>3</sub>CN. ESI-MS data was acquired on an Agilent LC/MSD-TOF instrument with a mass accuracy of 20 ppm and a range of 100 - 3500 Daltons. Calibration drift was minimized on a scan-by-scan basis by using internal standards corresponding to 922.0001 and 2721.0201 marker ions infused concurrently through a second nebulizer in the ionization chamber. Deconvolution was performed using on-board Agilent MassHunter software.

**Animal Experiments:** All experiments were performed in accordance with animal care protocols approved by the University of Kansas IACUC Committee. Cohorts of 5 outbred CF-1 mice per group were immunized on Day 0 with vehicle (control 1), 50  $\mu$ g/animal of bovine  $\alpha$ -lactalbumin alone (control 2), or  $\alpha$ -lactalbumin covalently coupled with 5 equivalents of **2**, or  $\alpha$ -lactalbumin mixed with 5 equivalents of **1** (control 3). All antigen preparations were in sterile, physiological saline (vehicle). A volume of 0.2 mL was injected intramuscularly into the flank region. Animals were boosted once on Day 14, and bled by terminal cardiac puncture (under isoflurane anesthesia) on Day 21. Sera were obtained from clotted blood by centrifugation at 3000g X 10 min, and stored at -80 °C until assayed.

**Enzyme-linked immunosorbent assays (ELISA):** A precision 2000 liquid handler (Bio-Tek, Winooski, VT) was used for all serial dilution and reagent addition steps, and a Bio-Tek ELx405 384-well plate washer was employed for plate washes; 100 mM phosphate-buffered saline (PBS) pH 7.4, containing 0.1% Tween-20 was used as wash buffer. Nunc-Immuno MaxiSorp (384-well) plates were coated with 30  $\mu$ L of  $\alpha$ -lactalbumin in 100 mM carbonate buffer, pH 9.0 overnight at 4 °C. After 3 washes, the plates were blocked with 3% bovine serum albumin (in PBS, pH 7.4) for 1 h at rt. Serum samples (in quadruplicate) were serially diluted in a separate 384-well plate using the liquid handler.

After three additional washes of the assay plate, 30  $\mu$ L of the serum dilutions were transferred using the liquid handler, and the plate incubated at 37 °C for 2 h. The assay plate was washed three times, and 30  $\mu$ L of 1:10,000 diluted appropriate anti-mouse immunoglobulin (IgG [ $\gamma$  chain], IgM [ $\mu$  chain], IgG1, IgG2a) conjugated with horseradish peroxidase was added to all wells. Following an incubation step at 37 °C for 1 h, and three washes, tetramethylbenzidine substrate was added at concentrations recommended by vendor (Sigma). The Chromogenic reaction was terminated at 30 min by the addition of 2M H<sub>2</sub>SO<sub>4</sub>. Plates were then read at 450 nm using a SpectraMax M4 device (Molecular Devices, Sunnyvale, CA). Data visualization and statistics (Student's T test for significance) were performed using Origin 7.0 (Northampton, MA).



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